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On the Cover

Visualization of zinc deposition and penetration using imaging mass spectrommetry of biofilms cross-sections. Heat mapping reveals highest zinc presence (red color) in biofilms treated with Dual Zinc plus Arginine (DZA) dentifrice..

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Changing the Paradigm of Daily Prevention: An Advanced Fluoride Toothpaste with Dual Zinc plus Arginine for Whole Mouth Protection and Whole Mouth Health

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Introduction

Carefully designed oral care products, which address the shortcomings of an individual's daily oral hygiene regimen, can significantly improve the effectiveness of oral hygiene regimens and contribute to improving oral health with minimal change in behavior. For this reason, an individual's choice of oral care products really matters if he/she is to achieve optimal oral health outcomes, such as maintaining good oral health or preventing specific oral health problems across life stages. By way of example, toothbrush features, such as head size, bristle design, and handle ergonomics, as well as brushing habits, such as method used and time spent tooth brushing, can each contribute to an individual's effectiveness of mechanical plaque removal.[1-4] Similarly, the inclusion of specially formulated, clinically proven ingredients is important to making a choice of toothpaste, as each can significantly improve the oral health outcomes both of the user wishing to maintain good oral health and the user experiencing a specific oral health problem or concern.[5-8] Making an informed choice that helps an individual to achieve his or her personal oral health goals is a challenge. Researchers can help by developing objective scientific and clinical evidence of product performance, and the dental profession can help by providing individualized product recommendations based on their assessment of this evidence.

The Colgate-Palmolive Company has embarked on a journey to change the paradigm of daily prevention. In seeking a truly innovative approach to support this move, Colgate has leveraged the recent change in focus of the dental profession from treatment of disease to active prevention, harnessed the trend to more health-conscious and educated patients and consumers, responded to contemporary demands to achieve and maintain good oral health, built upon a new understanding of oral biology and oral health, and exploited advances in technical innovation. In so doing, Colgate has created and validated a new "holistic" toothpaste that meets today's patient and consumer needs and, at the same time, embraces modern concepts and contemporary approaches to daily prevention.

This Special Issue publication provides a comprehensive overview of the scientific rationale for changing the paradigm of daily prevention and oral health and, thus, for developing and validating an advanced toothpaste to support this paradigm change. The papers in this Special Issue also provide scientific evidence of this new toothpaste's multiple mechanisms of action and objective clinical evidence of this new toothpaste's performance. This advanced fluoride toothpaste, with its Dual Zinc plus Arginine therapeutic active (0.96% zinc, as zinc oxide and zinc citrate, and 1.5% L-arginine), works differently from traditional antibacterial fluoride toothpastes which can start to work immediately and can significantly reduce bacteria between brushing occasions with continued use. The effects on bacteria of this new toothpaste build over time to subtly modulate the oral biofilm and help maintain symbiosis, *i.e.*, the natural balance of the oral microbiome, while also supporting the host defenses in the oral soft tissues. In other words, this new toothpaste exhibits the characteristics expected of an ecological approach to plaque control: it reduces bacteria to a lesser extent than traditional antibacterial toothpastes, and yet it delivers excellent efficacy in reducing plaque and gingivitis, as well as other plaque-related conditions, thus, offering multiple oral health benefits to its users when used on a regular and continued basis during routine tooth brushing.

Specifically, the fluoride strengthens teeth, and prevents enamel and root caries, while helping to prevent tooth erosion by food acids. Zinc was selected for its important role in many physiological and metabolic processes, including defense against oxidative stress, acceleration of wound healing processes, and the maintenance of immune function against pathogens, which are largely unexplored in oral health, as well as its antibacterial properties. The unique Dual Zinc plus Arginine therapeutic active was engineered to enhance delivery, retention, and bioactivity of zinc on soft tissues and deep into existing biofilm. When used regularly during routine tooth brushing, this new Dual Zinc plus Arginine fluoride toothpaste provides statistically significant reductions in oral biofilm on the teeth, tongue, cheeks, gingiva, and in saliva, and these reductions in oral biofilm are sufficient to deliver statistically significant and clinically meaningful reductions in dental plaque and established gingivitis. This new Dual Zinc plus Arginine fluoride toothpaste also provides statistically significant reductions in oral malodor through a combination of its antibacterial properties and complexing and neutralizing VSCs, and can reduce tartar build up by inhibiting crystallization. Finally, this advanced toothpaste is formulated with a high cleaning silica to whiten teeth and a small particle silica to reduce and prevent dentin hypersensitivity.

An Advanced Fluoride Toothpaste with Dual Zinc plus Arginine for 12-Hour Whole Mouth Protection and Whole Mouth Health: A Summary of Scientific and Clinical Studies

The first paper in this Special Issue is a review which provides a holistic framework for the development and validation of this advanced toothpaste that delivers whole mouth protection and whole mouth health. In so doing, it provides a historic perspective of the development of antibacterial oral care products and an overview of modern concepts and contemporary approaches to prevention and oral health.[9]

A comprehensive plaque control program designed, monitored, and reinforced by dental professionals can help a patient effectively

prevent dental caries and periodontal disease.[10] Informed, motivated individuals can achieve excellent standards of oral hygiene and oral health, but this approach is impractical for many individuals, with the result that dental caries and periodontal disease are prevalent on a global basis.[11-13] Patient-centered care offers a contemporary, holistic approach to overcome hurdles on the pathway to effective prevention and oral health, and ensures the long term success of dental professionals and healthy patients.[14] Use of a therapeutic toothpaste to supplement mechanical oral hygiene is also a pathway to enhance prevention and improve oral health. Experts recently acknowledged the importance of mechanical plaque control but, to overcome its practical limitations, recommended use of an adjunctive therapy with fluoride plus an antibacterial agent to simultaneously prevent dental caries and periodontal disease.[15-19]

A new definition of oral health has increased awareness of the different dimensions of oral health and has empowered dentistry to shift focus from treatment to support for prevention and oral health.[20] New understanding of oral biofilms in health and disease has shown that health-associated biofilms have a diverse flora comprising genera and species in a mutually beneficial relationship, known as symbiosis. They create a hostile environment to prevent the establishment of pathogenic species, and they help regulate the host response to prevent damage. In so doing, these health-associated biofilms provide essential benefits to oral health and well-being. Physiological or behavioral changes can impact a healthy biofilm and trigger dysbiosis with concomitant increases in pathogenic species present. These changes drive disease processes so effective interventions are needed to reestablish symbiosis and promote oral health.[15,21] An ecological approach to preserve symbiosis and prevent conditions leading to dysbiosis has been proposed. This approach has the potential of reducing bacteria in oral biofilm sufficiently to reduce disease risk, while creating and supporting the beneficial functions of oral biofilms consistent with health.[22] Oral biofilm on soft tissues is a reservoir of bacteria for recolonization of the teeth. Reducing oral biofilm on the soft tissues results in improvements in plaque control and oral health.[23-25] Thus, regular and continued use of an antibacterial fluoride toothpaste which provides users with 12-hour whole mouth protection and improved whole mouth health, demonstrated as positive outcomes on dental caries and gingivitis, as well as whiter teeth, fresher breath and absence of tooth sensitivity, could be argued to be the future cornerstone of prevention and good oral health.

As background to the selection of the Dual Zinc plus Arginine therapeutic active in this toothpaste, an overview of zinc as an essential trace element is provided with specific emphasis on zinc's untapped potential to deliver multiple simultaneous oral health benefits through tailored zinc chemistry and unexplored biological pathways. [26-29]

To meet today's professional, patient, and consumer needs, a holistic approach to effective prevention and good oral health is needed. Based on ecological approaches to oral biofilm management, this advanced fluoride toothpaste with Dual Zinc plus Arginine offers dental professionals and their patients, as well as consumers in general, the opportunity to take charge of their oral hygiene regimens through a pro-active choice of the toothpaste they use, and achieve whole mouth protection against future oral challenges and whole mouth health. As part of a risk-based preventive program which embraces patient-centered care, this toothpaste can empower patients to achieve effective prevention and good oral health. In addition, with direct-to-consumer messaging that motivates and empowers consumers to improve self-care, this toothpaste can also enhance prevention and improve oral health for anyone who uses it.[9]

The second paper in this Special Issue summarizes the science underpinning the selection of the Dual Zinc plus Arginine therapeutic active system. It describes studies which explored combinations of zinc citrate and zinc oxide, with and without L-arginine, to optimize the chemistry of the zinc system (zinc speciation) in the toothpaste and its delivery to relevant *in vitro* surfaces, *i.e.*, hydroxyapatite discs, Vitro™ skin and Epigingival™ tissue, and oral bacteria. The results of these studies showed that combinations of soluble and insoluble zinc enhanced the uptake of zinc to these surfaces. The addition of L-arginine to zinc citrate plus zinc oxide further increased uptake to the test surfaces compared to the dual zinc system alone, supporting the hypothesis that the Dual Zinc plus Arginine system provides an optimal combination of immediate release of zinc ions followed by a steady dissolution of insoluble zinc by intra-oral ligands over time. The paper also describes biological studies performed to confirm the selection of the Dual Zinc plus Arginine system as the form of zinc to overcome potential barriers to delivery, biofilm penetration, and retention in the mouth. Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were used as measures of bacterial growth and viability, whereas matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy (MALDI TOF MS) and confocal laser scanning microscopy (CLSM) were used to quantify delivery and image the location of zinc uptake. The results of these studies support that the Dual Zinc plus Arginine system enhanced the delivery of zinc and its effectiveness in reducing bacterial growth and metabolism, thus confirming the importance of zinc dose and form (zinc speciation) on bioavailability and activity of zinc as a therapeutic antibacterial agent.[30]

Outstanding toothpaste aesthetics are of critical importance to patients' and consumers' compliance with usage and, ultimately, to their success in improving oral health. The third paper in this Special Issue describes the art and science of developing a flavor that is stable for the shelf life of the Dual Zinc plus Arginine fluoride toothpaste, masks both the astringency and metallic taste of zinc, is well liked by consumers, stimulating compliance to product usage instructions and the delivery of promised oral health benefits, and ensures continued brand loyalty for Colgate's therapeutic toothpaste segment. The stability of the toothpaste with test flavors was analyzed by gas chromatography-flame ionization detection and gas chromatography-mass spectroscopy after aging, taste acceptability was assessed by a flavor expert using organoleptic scores, and consumer acceptance was validated in monadic identified product tests in key markets globally. Based upon the scientific learning from these in-depth studies, flavors that are stable, consumer appealing, and consistent with therapeutic branding were identified.[31]

The fourth paper in this Special Issue describes the results of a randomized, single-center, three-cell, double-blind, parallel-group clinical study conducted in Karnataka, India in which three toothpastes, one containing the Dual Zinc plus Arginine active and 1000 ppm fluoride as sodium fluoride, another containing the Dual Zinc plus Arginine active and 1450 ppm fluoride as sodium fluoride, and a third containing 1450 ppm fluoride as sodium fluoride, were compared for their effects on oral bacteria collected from multiple soft and hard tissue sites, as well as saliva, 12-hours after 14 and 29 days

of twice-daily brushing. Small, but statistically significant reductions in numbers of bacteria on the teeth, tongue, buccal mucosa, and gingiva, and in saliva, were observed for both Dual Zinc plus Arginine fluoride test toothpastes compared to the regular fluoride toothpaste control, 12 hours after 29 days of product use. Qualitatively similar results were observed after just 14 days of product use. The two Dual Zinc plus Arginine fluoride test toothpastes were shown to be statistically equivalent.[32] The results of this study are consistent with the proposed mechanism of action of the Dual Zinc plus Arginine active, *i.e.*, modulation of the oral biofilm through subtle antibacterial mechanisms, coupled to positive effects on biological processes within the host response.

The fifth paper in this Special Issue describes the results of a randomized, single-center, two-cell, double-blind, parallel-group clinical study conducted in Santo Domingo, Dominican Republic in which a toothpaste containing Dual Zinc plus Arginine and 1450 ppm fluoride as sodium fluoride, and a toothpaste containing 1450 ppm fluoride as sodium fluoride, were compared for their effects on dental plaque (Plaque Index, PI; Plaque Severity Index, PSI; and Plaque Interproximal Index, PII) and gingivitis (Gingivitis Index, GI; Gingivitis Severity Index, GSI; and Gingivitis Interproximal Index, GII) after three and six months of twice-daily brushing. Of the 100 subjects who entered the study, 96 complied with the protocol and completed the study. Statistically significant reductions ($p < 0.001$) were observed for both products on all clinical measures at both time points. Importantly, statistically significant reductions ($p < 0.001$) in PI, PSI, and PII of 30.1, 61.9, and 28.0%, respectively, and in GI, GSI, and GII of 26.3, 56.6, and 29.2%, respectively, were demonstrated for the Dual Zinc plus Arginine fluoride test toothpaste compared to the regular fluoride toothpaste control after six months of product use.[33]

The sixth paper in this Special Issue describes the results of a randomized, single-center, two-cell, double-blind, parallel-group clinical study conducted in Chengdu, China in which a toothpaste containing Dual Zinc plus Arginine and 1450 ppm fluoride as sodium fluoride was compared to a toothpaste containing 1450 ppm fluoride as sodium fluoride, for their effects in controlling oral malodor 12 hours (overnight) following three weeks of twice-daily brushing. Eighty subjects were enrolled, complied with the study protocol, and completed the study. At the start of the study, organoleptic scores were rated as moderately to very unpleasant (7.35 and 7.34) for test and control, respectively. Statistically significant ($p < 0.001$) reductions in organoleptic scores were observed for both groups 12 hours (overnight) following three weeks of twice-daily use, at which time the users of the Dual Zinc plus Arginine fluoride toothpaste had improved sufficiently to be scored as having neutral to slightly pleasant breath (score = 4.49), whereas the users of the regular fluoride toothpaste control were scored as having slightly to moderately unpleasant breath (score = 6.49). This represented a statistically significant difference ($p < 0.001$) of 30.8% in favor of the Dual Zinc plus Arginine fluoride test toothpaste compared to the fluoride control.[34]

The final paper in this Special Issue describes the results of two clinical studies on oral malodor. One study was a four-cell, double-blind, parallel-group study in which the Dual Zinc plus Arginine toothpaste with 1450 ppm fluoride as sodium fluoride was compared to a toothpaste containing 1450 ppm fluoride as sodium fluoride, as well as two experimental toothpastes, for their effects in controlling oral malodor after one, two, and four weeks of twice-daily brushing. The second study was a two-cell, double-blind, cross-over study in which the Dual Zinc plus Arginine toothpaste with 1450 ppm fluoride as sodium fluoride was compared to a toothpaste containing 1450 ppm fluoride as sodium fluoride for their effects in controlling oral malodor, 12 hours (overnight) after a single use. In both studies, oral malodor was scored using organoleptic and hedonic scores. In addition, volatile sulfur compounds (VSCs) were measured in the second study using a new technique known as selected ion flow mass spectroscopy, used for the first time to study the effects of toothpastes on oral malodor. Statistically significant differences in organoleptic and hedonic scores were observed in favor of the Dual Zinc plus Arginine toothpaste in the two studies. These differences increased with extended use in the four-week study, shifting the population to healthier breath. A statistically significant reduction in VSCs was also observed in the single-use study.[35]

Conclusion

By choosing this new, advanced toothpaste containing Dual Zinc plus Arginine (0.96% zinc, as zinc oxide and zinc citrate, and 1.5% L-arginine) and 1450 ppm (or 1000 ppm) fluoride as sodium fluoride, users are empowered to enhance the effectiveness of their tooth brushing routine, with just a simple behavioral change, to achieve more effective prevention and improved oral health outcomes that meet their personal oral care needs.

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Conflict of Interest: Dr. D. Cummins is a retiree of the Colgate-Palmolive Company. She is currently an independent consultant and was funded by the Colgate-Palmolive Company to author this paper.

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Changing the Paradigm of Daily Prevention to Achieve Whole Mouth Health in the 21st Century

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Abstract

A comprehensive mechanical plaque control program – designed, monitored, and reinforced by dental professionals – can help patients achieve excellent oral hygiene and oral health. However, this approach to prevention is impractical for many individuals, so dental caries and periodontal disease are highly prevalent globally. Experts recently agreed that a toothpaste with fluoride and a plaque control agent can augment mechanical procedures to simultaneously prevent caries and periodontal disease. Notwithstanding this, it is timely to rethink prevention and oral health promotion.

A new definition of oral health raises awareness of its different dimensions and empowers dentistry to move from treating disease to supporting prevention. In addition, a deeper understanding of the relationship between oral biofilms and the host facilitates new opportunities for disease prevention. The knowledge that health-associated biofilms help prevent establishment of pathogenic species, regulate the potentially damaging host response, and provide essential benefits to health and well-being is paramount to changing the paradigm of prevention of dental disease. Ecological approaches to biofilm control can reduce plaque sufficiently to reduce disease risk, while creating and supporting the beneficial functions of biofilms consistent with health. The knowledge that the oral soft tissues are the primary reservoir of bacteria for tooth recolonization and that reducing bacteria on soft tissues results in improved plaque control and consequently better oral health is also salient. Indeed, a toothpaste that delivers 12-hour protection to the hard and soft tissues (whole mouth protection) and multiple oral health benefits (whole mouth health) could become the future cornerstone of prevention.

An innovative fluoride toothpaste with a Dual Zinc plus Arginine multi-functional therapeutic agent offers whole mouth protection against daily oral challenges and whole mouth health for the patient. Within a patient-centered preventive program, next to messaging that motivates towards improved self-care, this toothpaste empowers patients to achieve effective prevention of common oral diseases and better oral health compared to just brushing with an ordinary toothpaste.

(J Clin Dent 2018;29(Spec Iss A):A1–9)

Introduction

The pioneering clinical studies on prevention in oral health initiated by Axelsson and coworkers in the 1970s, and reported after 6, 15, and 30 years of monitoring, demonstrate that a comprehensive dental plaque control program can help individuals successfully prevent and control dental caries and periodontal disease.[1-3] The program comprised professional dental cleanings (at 2- to 12-month intervals according to individual needs) and meticulous self-care (mechanical plaque removal through twice-daily tooth brushing and daily interdental cleaning), supported by on-going coaching by a dental professional (on an individual needs basis) and education in self-diagnosis and self-care.[1-3] Today, such an intense and personalized dental plaque control program might be considered patient-centered care.

Patient-centered care is a relatively new term in medicine and dentistry and refers to provision of care that is respectful of, and responsive to, individual patient needs, preferences, and values, and provides a guide for clinical decision making. Patient-centered care is a dimension of quality within health care, and the delivery of patient-centered care is a long-term vision for all patients everywhere.[4] From a patient perspective, patient-centered care equates to enhanced satisfaction, improved health outcomes, enhanced health status, and reduced need for treatment. From a professional point of view, patient-centered care equates to increased satisfaction and reduced risk of dissatisfied patients and potential litigation.[4] Today, patient-centered care is less well developed in dentistry than in medicine, yet the concepts and benefits to patients and dental professionals are equally tangible. Core to successful patient-centered care in dentistry are respectful dentist-patient relationships, mutual trust, patient involvement, individualized care, humanistic non-judgmental communication, empathy and understanding, and quality information and support.[4] Patients also identify patient-centered care with the delivery of high-quality care. They value the connection to, and attitude of, the dental professional team, good communication through two-way exchange of information, empowerment through provision of choice and independent decision making, and feeling valued as an individual whose opinions and views are appreciated and beliefs and circumstances are respected.[5] An increasingly important element of patient-centered care in medicine and dentistry is a strong focus on disease prevention. In dentistry, there is no doubt that a conscious shift in focus to individualized professional care, motivated self-care, and prevention of oral health problems will ensure the long-term success of dental professionals and increasingly healthy patients throughout life. Interestingly, the evolution of dentistry to a patient-centered care

approach is consistent with the more revolutionary new field of proactive P4 (predictive, preventive, personalized, and participatory) medicine, which is emerging from the convergence of systems biology and medicine with the digital revolution.[6] Patient-centered care and prevention in dentistry will likely gain impetus from P4 medicine's success.

Informed and motivated individuals are capable of achieving the excellent standards of oral hygiene and oral health observed in the Axelsson study. However, at the population level many individuals are unable to follow the stringent procedures needed to achieve outstanding mechanical plaque control on a routine basis, with the result that dental caries and periodontal disease remain prevalent and raise public health concerns on a global basis.[7-12] Without doubt, there are many factors contributing to this situation, some of which are especially pertinent to the arguments put forward in this paper. One factor is access to professional care. The dental profession has played an important role in transferring the knowledge that meticulous oral hygiene is a fundamental requirement for excellent oral health, and encouraging their patients to achieve effective mechanical plaque removal through twice-daily tooth brushing and daily interdental cleaning. The problem is that many individuals do not have regular access to the dental profession, may not fully appreciate the importance of effective tooth brushing, or may not have the skills required to brush meticulously, so they are unable to achieve complete plaque removal during routine tooth brushing.[13] In addition, interdental cleaning may not be routine for these individuals.

Another factor is the effectiveness of traditional chairside methods of oral health promotion. A systematic review determined the need to develop an effective model for chairside oral health promotion that encourages dental professionals to increase their focus on the social determinants of oral health and disease during the clinical encounter. It also identified an opportunity to further leverage behavior change methodologies for improved communication in the oral health arena.[14,15] The intervening years suggest that these needs and opportunities are still relevant. A third factor, also related to the effectiveness of chairside communication, is patient compliance. Dental professionals may invest time and effort in prevention, but patient compliance with professional oral health recommendations can be a major issue, simply because patients may perceive a negative message of "you have not properly taken care of your teeth or gums" from their dentist or hygienist. Also related is a fourth factor, *i.e.,* health literacy. Higher health literacy is associated with better patient-dentist communication, regular dental care, and, importantly, better self-rated oral health. This suggests that improved health literacy is one important pathway to better oral health.[16]

Another important pathway to better oral health is through use of therapeutic oral care products that supplement mechanical plaque control measures to ultimately prevent dental caries and periodontal disease. Toothpastes are ideal vehicles for the delivery of such therapeutic agents because they are ubiquitously used during routine tooth brushing and can be formulated with excellent aesthetics to aid user compliance with recommended brushing instructions.[17,18] Fluoride was the first therapeutic agent developed and validated to supplement mechanical plaque control for the prevention of dental caries. It targets the tooth surface to render enamel less susceptible to the deleterious effects of caries-causing oral bacteria in dental plaque. Toothpastes containing 1000–1500ppm fluoride are clinically proven to reduce cavities by 20–30%.[19] Their widespread adoption continues to play an important role in caries prevention, and has helped reduce the prevalence and severity of dental caries over the past several decades, albeit that challenges remain.[20] The development of therapeutic agents to supplement mechanical plaque control to prevent gingivitis has focused on antibacterial agents to reduce bacterial growth and metabolism and, thereby, reduce the detrimental effects of bacteria on the gingiva.[21] Several products containing antibacterial agents, including mouth rinses with either chlorhexidine, essential oils, or cetylpyridinium chloride,[22] and fluoride toothpastes with either triclosan/copolymer,[23] triclosan/zinc citrate,[24] or stannous fluoride,[25] have been shown to reduce plaque and prevent and control gingivitis. Recently published systematic reviews, meta-analyses, and consensus papers covering different aspects of the prevention of dental caries and periodontal disease have each acknowledged the important role and practical limitations of mechanical plaque control, and reaffirmed the value of supplementing mechanical oral hygiene measures with an adjunctive therapy combining fluoride and an antibacterial system to enable the simultaneous prevention of caries and periodontal diseases. Further, as prevention and treatment strategies are effective at all ages, this guidance is applicable for individuals across all life-stages.[26-30] Two characteristics of toothpastes containing therapeutic agents for the prevention of dental caries and gingivitis are critical to their effectiveness in clinical studies. The therapeutic agents in these products must be efficiently delivered to the hard and soft tissues in the mouth during brushing, and they must be retained on these surfaces and work effectively at low levels throughout the time period between oral hygiene occasions.[17,18,31]

Modern Concepts and Contemporary Approaches to Achieve Effective Prevention and Oral Health

A New Definition of Oral Health

Oral health was officially recognized in 2000 by the United States Surgeon General as an integral part of health and well-being.[32] However, in the intervening period, it has not been clear whether the term "oral health" has meant the same thing to different facets of the medical and dental profession and their many stakeholders. Without a clear and unambiguous definition of "oral health," the influence of the dental profession on oral health advocacy, oral health research and education agendas, oral health policies, and the future of the profession itself was felt to be diminished. For this reason, a new definition, which acknowledges the multi-faceted nature and attributes of oral health, was created and overwhelmingly approved in 2016 by the FDI World Dental Federation Assembly.[33,34] In essence, the definition states that "oral health includes the ability to speak, smile, smell, taste, touch, chew, swallow, and convey emotions through facial expressions with confidence and without pain, discomfort and disease of the craniofacial complex. It is a component of health and well-being and exists along a continuum influenced by the values and attitudes of people and communities. It reflects the physiological, social, and psychological attributes that are essential to quality of life and it is influenced by the person's changing experiences, perceptions, expectations, and ability to adapt to circumstances."[33,34] This new definition, together with its companion

framework, raises awareness of the different dimensions of oral health and, importantly, empowers dentistry to move from simply treating disease to providing care and support for prevention and the proactive promotion of oral health.[33,34]

The Role of Microbial Biofilms in Oral Health and Disease

Oral biofilms are widely recognized as the primary cause of the common oral diseases, dental caries and periodontal disease, and of the cosmetic concerns of calculus and oral malodor, illustrated in Figure 1. There is recent consensus among academic experts that effective biofilm management is a key strategy for the prevention of these diseases and conditions, and for successfully achieving oral health.[26,35,36]

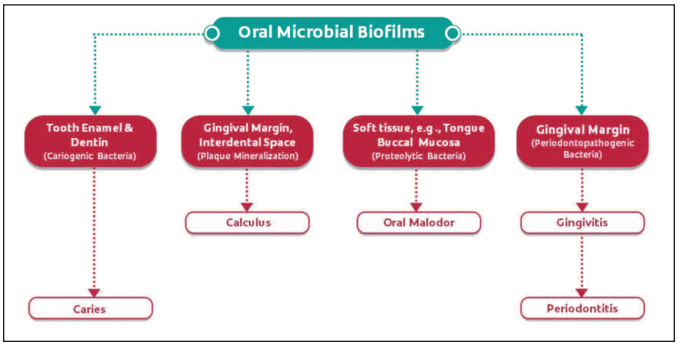

Figure 1. *Oral microbial biofilms are widely recognized as the primary cause of the common oral diseases, dental caries and periodontal disease, and of cosmetic concerns like calculus and oral malodor.*

Oral biofilms typically comprise a highly diverse group of bacteria that are attached to all oral surfaces and embedded in an extracellular matrix. Of the 750+ oral bacterial species that have been identified to date, between 100 and 300 species may be present in any one individual's mouth.[36] Bacteria in a biofilm are functionally distinct from their planktonic counterparts because the pattern of gene expression can be different when cells attach to a surface, and the genera and species present in a biofilm act in concert as an integrated community through a range of interactions that can be either synergistic or antagonistic, depending on the nature of the bacterial species and the specific environment in which they reside.[26,35-38] The properties of a biofilm are, therefore, more than the sum of the activities of the constituent species. The term "oral biofilm" does not describe a single state; it is a collective term describing a wide continuum of functional states and includes that oral biofilm composition and functionality can vary significantly from person to person, site to site, time to time, and life-stage to life-stage within any one individual. With respect to biofilm composition, there is a fine dividing line between health and disease. This is why, on the one hand, oral biofilm can be discussed as the main cause of common oral diseases, yet, on the other hand, can be discussed for its positive attributes in the oral cavity. For this reason, it has now become widely recognized and accepted that certain features of oral biofilms are critical to their respective roles in both health and disease.[23,32-35]

Recent studies have identified a core group of genera, including *Neisseria*, *Streptococcus*, *Veillonella*, *Granulicatella*, *Gemella*, *Prevotella*, *Rothia*, *Fusobacterium*, and *Actinomyces*, that are associated with oral health.[26,35] These bacteria are found naturally in the mouth, they are termed the "normal" oral microbiome and have a symbiotic relationship with the host; the host provides a favorable environment for microbial growth (warmth, nutrition, etc.) and the oral micro-organisms deliver critical functions that benefit the host. For example, oral biofilms act as a barrier and produce a hostile environment to prevent colonization by exogenous, and often pathogenic microbes, whereas some oral bacteria play an immunomodulatory role, down-regulating potentially damaging pro-inflammatory responses to beneficial organisms. Others are involved in regulating cardiovascular functions.

Thus, health-associated biofilms can provide essential benefits to the individual's health and well-being.[26,35-38] However, if the "normal" oral microbiome is perturbed by changes in an individual's physiology or behavior, an unnatural and unbalanced state, described as dysbiosis, can occur.[26,35-38] When conditions of dysbiosis persist, the composition of oral biofilms changes substantially. In diseased sites, oral biofilms harbor increased numbers of pathogenic species and lower proportions of the organisms associated with health, and they possess specialized metabolic feedback features that can perpetuate disease-favoring conditions.[26,35-38] In caries, the trigger of dysbiosis is frequent sugar exposure; these sugars are converted to acid by saccharolytic and acid-tolerant bacteria, such as mutans streptococci and lactobacilli, the organisms most commonly linked to caries. In turn, the acidic conditions are inhibitory to species associated with health, and the diversity of the oral microbiome decreases, accentuating the state of dysbiosis.[26,35-38] In gingivitis, the trigger is gingival inflammation. If the host response does not clear the microbial challenge, the altered environment favors the growth and persistence of proteolytic and obligately anaerobic species (recently called "inflammophiles"), that include such species as *Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia.* These species can also subvert and dysregulate the host's immune response, again lowering the proportion of organisms associated with health but, in contrast to caries, often expanding the diversity and richness of the subgingival microbiota.[26,35-38] In both dental caries and periodontal disease, the state of dysbiosis, illustrated in Figure 2, drives the development and progression of disease. This state of dysbiosis must be reversed, and

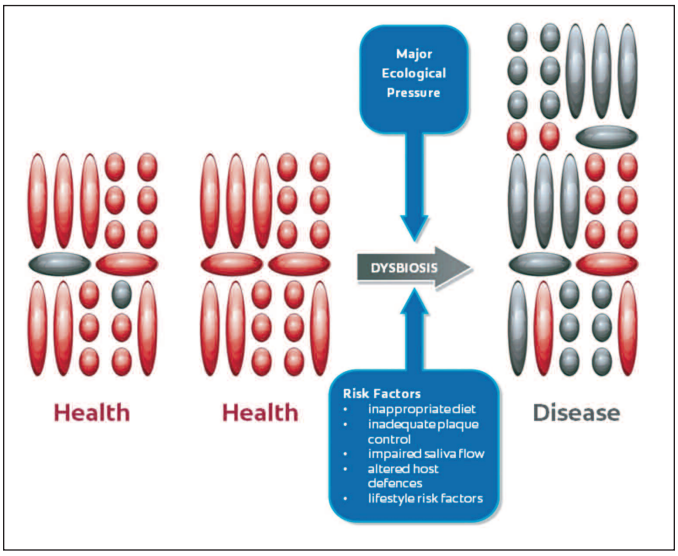

Figure 2. *A dysbiosis model, adapted from Marsh P D., Microbiology 2003; 149: 279–294. In health, the majority of bacteria have a symbiotic relationship with the host (symbiotic bacteria marked in red). Potentially pathogenic bacteria (marked in gray) can be present in health at low levels that are not clinically relevant. In disease, there is an increase in the overall number of bacteria as well as an increased proportion of pathogenic bacteria.*

symbiosis and homeostasis re-established through effective interventions to prevent disease processes and re-establish oral health.[38,39]

An Ecological Approach to Effective Plaque Control and Oral Health

In the light of the current understanding of the role of oral biofilm in oral health and disease, views on the use of antibacterial agents to supplement mechanical plaque control to achieve effective plaque control and the maintenance of good oral health have been contemporized in an ecological approach. This approach is aimed at preserving the symbiotic state of the normal oral microbiome and preventing conditions that lead to dysbiosis.[37-39] Specifically, it is proposed that antibacterial oral care products should sufficiently reduce microbial load in order to reduce the risk of dental disease, while creating and supporting the beneficial functions of the "normal" oral microbiome consistent with health. In so doing, these antibacterial products would likely control dental plaque and promote oral health through subtle effects on oral biofilms that last in between brushing occasions. Thus, in addition to providing statistically significant clinical reductions in plaque, gingivitis, and other related benefits, a newly developed antibacterial oral care product, based on this ecological approach, could offer patients and consumers extra protection against future detrimental physiological changes with a simple change from their regular toothpaste to a more effective product.[38,39]

The Concept of Whole Mouth Protection as the Future Cornerstone of Prevention and Whole Mouth Health

Researchers and dental professionals most often discuss oral biofilms in the context of their presence on and around the tooth surfaces, and their role in these locations in driving initiation and progression of dental caries and periodontal disease. However, it is important to appreciate that the teeth only account for ~20% of the total surface area of the mouth, with the soft tissues accounting for four times the surface area of the teeth, *i.e.*, ~80% (Figure 3, left).[40] Equally important is the fact that oral bacteria are distributed throughout the mouth in proportion to the surface area of the hard and soft tissues; just 20% of the total oral biofilm is on the teeth and 80% is on the soft tissues in diverse locations throughout the mouth, including the tongue, buccal and gingival mucosa (Figure 3, right).[41]

What is the relevance of knowing that oral bacteria colonize both the hard and soft tissues in proportional amounts to their surface areas? A reasonable response to this question might be: "Even for those individuals who exhibit good mechanical plaque control through effective tooth brushing and interdental cleaning, there is a significant reservoir of bacteria within biofilms on the soft tissues that can be shed into saliva and transferred to recolonize the teeth." This response is both plausible and justified,[26] and poses a testable hypothesis: "If individuals could reduce bacteria throughout their mouths, not just in the biofilm on their teeth but also in the biofilms on their tongues, cheeks, and gums, would dental plaque, gingivitis, and other plaque-related oral health measures be improved?" The short and simple answer to this question is "Yes" and there are data to support this hypothesis, *vide infra*.

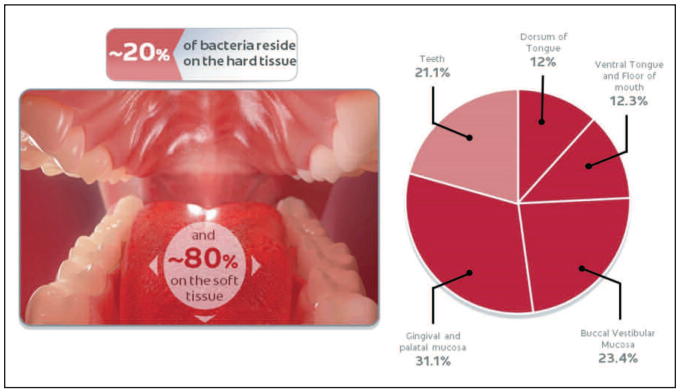

Figure 3. *Soft tissue accounts for 80% of the oral surfaces (Collins LM, Dawes C.* J Dent Res *1987;66:1300-02). Oral bacteria are distributed throughout the mouth in proportion to the surface areas of the hard and soft tissues, with the majority being harbored on the soft tissues in diverse locations* (*Mager DL,* et. al. J Clin Periodontol *2003;30:644-54*).

First, an example of mechanical plaque control that demonstrates this hypothesis to be true should be considered. Clinical studies have demonstrated that brushing with a specially designed toothbrush with a tongue cleaning implement to remove bacteria from the tongue, cheeks and gums, in addition to the teeth, provides statistically significant reductions in dental plaque and gingivitis, as well as in oral malodor (volatile sulfur compounds, or VSC's, and hydrogen sulfide-producing bacteria) compared to brushing with a conventional toothbrush that removes plaque from the teeth alone.[42-45] The use of an antibacterial toothpaste to supplement mechanical plaque control further demonstrates this hypothesis to be true. Brushing with a fluoride toothpaste containing triclosan/copolymer was shown to provide statistically significant reductions in bacteria on the teeth, tongue, cheeks, and gums 12 hours after brushing, as well as statistically significant reductions in dental plaque, gingivitis, and other oral health parameters compared to brushing with a regular fluoride toothpaste.[23,46,47] These two examples clearly demonstrate that reducing bacteria on the hard and soft tissues, either by utilizing novel mechanical procedures or by delivering 12-hour whole mouth antibacterial protection, results in statistically significant reductions in dental plaque, gingivitis, and related oral health measures.[23,42-47]

Considering the wide accessibility and ease of use, daily brushing with an effective antibacterial fluoride toothpaste, which provides 12-hour whole mouth protection and improved whole mouth health (demonstrated as positive outcomes on dental caries and gingivitis), as well as whiter teeth, fresher breath, and absence of tooth sensitivity, could be argued to be the future cornerstone of active prevention and good oral health.

Zinc: An Essential Trace Element with Untapped Potential to Deliver Multiple Simultaneous Oral Health Benefits through Tailored Zinc Chemistry and Unexplored Biological Pathways

It has recently been stated that "A diet rich in essential nutrients and anti-oxidants is indispensable to a healthy mind and body."[48] There is no doubt this statement is true for zinc, it being both an essential nutrient and an anti-oxidant. Zinc has enjoyed a range of uses as a therapeutic in medicine, two interesting and potentially relevant examples of which are the use of zinc to shorten the duration of the common cold and the use of zinc to improve wound healing.[49-51] Although not yet widely recognized, these examples have illustrated to the medical profession that the dosage and form of zinc used in a medicinal product are critical to whether or not it is effective in delivering a specific health

benefit. In summary, zinc must be delivered in a stable, labile (available), and metabolically active form to effectively modulate cell physiology (prokaryote and/or eukaryote as appropriate to the specific application) for positive health benefits.[51]

Zinc is an essential trace element and is ubiquitous across all life forms. It plays a vital role throughout the human body in maintaining numerous physiological and metabolic processes in living tissues, such as defense against oxidative stress, including protection against aging and acceleration of wound healing processes, support of regenerative processes, including DNA and protein synthesis, and maintenance of immune function against pathogens.[48,50,51] These processes, however, have been largely unexplored with regard to specific research on the natural function of zinc in the oral cavity and its role in establishing and preserving oral health. Zinc is the second most abundant metal in the human body and is the only one that impacts all three major functions of enzymes: catalysis, regulation, and structural integrity.[48,50,51] Zinc is naturally located in several of the organs and tissues throughout the human body, including the skin. While published research on the presence of zinc in the oral soft tissues is currently limited, by analogy to the skin one can assume that zinc is naturally present in the oral mucosa and soft tissues, and that it plays an important role in the physiological and metabolic processes in the mouth. In contrast, there is published research demonstrating zinc's presence in enamel and dentin, as well as in dental plaque and saliva.[52]

Fluoride toothpastes containing zinc have been marketed for several decades. Zinc was first recognized for its antimicrobial properties, and toothpastes containing zinc citrate were investigated and shown to help reduce bacteria that are responsible for dental plaque consistent with their antibacterial properties.[53,54] Zinc citrate was selected because it was stable in toothpaste formulations and yet sufficiently labile in the mouth to deliver elevated levels of zinc to dental plaque and saliva, where it was shown to be retained after brushing.[31,54] A collaboration between chemists and microbiologists identified the importance of zinc speciation, and provided an initial understanding of the relationship between the chemistry of zinc and its biological activity as an antibacterial agent. Studies demonstrated that free zinc [Zn_{aq}^{2+}] was the most effective form of zinc for antibacterial activity, and indicated that positively charged and neutral complexes of zinc with amino acids may play an active role in delivering anti-plaque effects *in vivo*, the latter being of special interest to this paper.[55-58] Thus, these studies clearly showed that the availability (dosage and form) of zinc was critical to the clinical effectiveness of zinc-containing oral care products, in line with the more recent observations in the medical field. On the basis of *in vitro* studies, potential mechanisms of antibacterial action were proposed which include, but are not limited to, inhibition of nutrient uptake and bacterial metabolism, inhibition of adhesion to oral surfaces, and enhancement of bacterial aggregation for enhanced clearance of bacteria by saliva.[52,60-63] Recent clinical studies have confirmed that zinc is delivered effectively from zinc citrate-based fluoride toothpastes to both hard and soft tissue surfaces in the mouth, where it displays substantivity and antibacterial protection after brushing.[64,65] Clinical studies have also confirmed that brushing with a zinc citrate-based fluoride toothpaste provides statistically significant reductions in plaque and gingivitis compared to brushing with a regular fluoride toothpaste.[66-68] In general, clinical studies have evaluated the effects of an antibacterial fluoride toothpaste in improving gingival health, including the three reported above.[66-68] An alternative approach is to use an antibacterial fluoride toothpaste to maintain good oral health following professional dental care.[69,70] This "alternative" approach has been demonstrated using a zinc-based fluoride toothpaste to help maintain gingival health over a 3-month period post professional care.[71,72]

Additional oral health benefits of zinc citrate toothpastes have been demonstrated. Oral malodor is generated as a result of bacterial metabolism of proteins and amino acids present in oral debris after eating and drinking. VSCs, such as hydrogen sulfide and methyl mercaptan, are particularly noxious. Brushing with a zinc-containing fluoride toothpaste provides statistically significant reductions in oral malodor compared to brushing with a regular fluoride toothpaste.[73] *In vitro* studies indicate that zinc can prevent and control oral malodor by reducing the number of odor-causing bacteria and by neutralizing the VSCs they generate.[74] In addition to possessing antibacterial properties, zinc citrate can inhibit crystal growth. Brushing with a zinc citrate-based fluoride toothpaste provides statistically significant reductions in the buildup of dental calculus compared to brushing with a regular fluoride toothpaste.[75,76] Based on *in vitro* studies, zinc has also been suggested to augment the effects of fluoride in reducing dental caries by inhibiting enamel demineralization and modifying remineralization, but clinical efficacy for such benefits still needs to be documented.[77]

In summary, zinc-based fluoride toothpastes have shown effects in reducing bacteria within oral biofilms, in preventing and controlling plaque and gingivitis, in reducing oral malodor, and in preventing calculus build up. However, there are opportunities to improve upon historic zinc-based formulas, as well as new avenues of unexplored potential. In respect to opportunities for improvement, zinc-based (and stannous-based) toothpastes may impart a metallic or astringent taste to their users. It is, therefore, important to formulate a new zinc-based toothpaste with a flavor that can effectively mask metallic taste and astringency and deliver excellent esthetics. There is also opportunity to build upon published knowledge of zinc speciation to engineer the form and dosage of zinc used in a new toothpaste for optimal delivery and retention, as well as the bio-availability of zinc in the oral cavity. Such optimization has the potential to increase the effectiveness of the zinc as an antimicrobial, as well as to overcome possible regulatory mechanisms by which exogenous soluble zinc is controlled by the host. In respect to new avenues of unexplored potential, there is opportunity to explore the ability of zinc to subtly modulate the composition or functionality of the oral biofilm and help maintain the natural balance of the mouth through an ecological approach to oral health prevention. There is also opportunity to explore the effects of exogenous zinc on the broad range of natural physiological and metabolic processes occurring in oral tissues, especially in the support of regenerative processes and maintenance of immune function against oral pathogens.

L-arginine: A Semi-essential Amino Acid with Proven Oral Health Benefits and Untapped Potential to Enhance the Delivery and Functionality of Zinc

L-arginine has grown in interest as a result of its inherent chemical and biological functionalities. The following section summarizes how L-arginine has successfully been leveraged for these properties to effectively treat both caries by modulating the pH of plaque

biofilm, and hypersensitivity through delivery of calcium into dentin tubules. Based on these disparate modes of action, the authors believe L-arginine may be further leveraged in combination with the appropriate dentifrice formulation to deliver additional benefits in the oral cavity.

L-arginine is a unique multifaceted amino acid with the potential to play one or more independent roles in controlling biofilm, influencing physiological processes in the mouth, and consequently preventing dental disease. L-arginine plays a direct role in preventing dental caries by altering the plaque metabolism and compositions.[78,79] As mentioned earlier in this paper, a number of species, naturally present in oral biofilms, have dual functionality as acid-producing and acid-tolerant species, whereas different species in oral biofilms have complementary alkali-producing functionality.[80] Dental caries is triggered by factors leading to an increase in the acid-producing capacity and prevented and controlled by factors leading to an increase in alkali-producing capacity of the biofilm.[81] More recent research on the mechanisms of alkali production and their impact on caries status has confirmed the importance of this mechanism in controlling oral biofilm and dental caries.[79,82-85] This mechanism is the mouth's natural defense against dental caries, and is fundamental to controlling the environment within the oral biofilm and to preventing change from a healthy to a pathogenic'state.[86] Modulation of this mechanism by subtle manipulation of the functionality of the oral biofilm, such as increasing its arginolytic capacity, is one example of an ecological approach to biofilm control, of high relevance for the prevention of dental caries.

The documented efficacy of a fluoride toothpaste containing 1.5% L-arginine and an insoluble calcium compound for the prevention of dental caries supports the validation of L-arginine as a valuable component of oral care products. The role of the exogenous L-arginine is to subtly modulate plaque metabolism and increase plaque pH through the production of ammonia by the resident (and natural) arginolytic species in the oral biofilm. The insoluble calcium compound provides a source of calcium ions, poised at slightly alkaline pH, for the remineralization of demineralized tissue. Several randomized clinical trials (RCTs) using different clinical protocols to determine the *in vivo* efficacy of the fluoride toothpaste containing L-arginine and an insoluble calcium compound proved that this particular formulation provides superior efficacy in preventing dental caries in comparison to a regular toothpaste with fluoride alone.[87]

L-arginine also plays a role in providing relief of dentin hypersensitivity in combination with an insoluble calcium compound. Dentin hypersensitivity can occur when gums recede or when enamel is worn away over time and exposes the underlying dentin surface and dentin tubules to the external environment. External stimuli such as hot, cold, or pressure cause the fluid residing within the dentin tubule to expand or contract, which is relayed to the nerve endings in the pulpal chamber and triggers a short, sharp pain response. Research into the natural mechanism of blocking exposed dentin tubules revealed that this mechanism can be enhanced through *in situ* formation of insoluble calcium phosphate within the open and exposed tubules, forming a dentin-like plug which blocks pain triggers.[88]

The efficacy of a fluoride toothpaste containing 8% L-arginine and calcium carbonate for the treatment of dentin hypersensitivity, demonstrated in a series of RTC's,[88] further documents the benefit of including L-arginine in oral care products. In such a formulation, the exogenous L-arginine and the insoluble calcium compound provide a source of calcium ions which, at slightly alkaline pH, can instantly precipitate as calcium phosphate within exposed dentin tubules to block and seal them.

As illustrated in the preceding examples, arginine is truly unique and provides oral health benefits through chemical and biological modes of action. It has recently been discovered that L-arginine may enhance the penetration and retention of antimicrobial agents in biofilms. Multiple mechanisms likely explain the tolerance to antimicrobial agents of bacteria in biofilms relative to their planktonic states. These include slow growth rate, induction of stress response, development of a biofilm-specific biocide-tolerant phenotype, and binding to or quenching of the agent by the biofilm matrix, thereby reducing penetration.[90,91] L-arginine has been shown to disrupt and destabilize biofilm integrity and inhibit the formation of exopolysaccharide (EPS).[92,93] Further, L-arginine has been shown to increase penetration of CPC and increase its ability to kill bacteria in multispecies biofilms, attributed to its effects on biofilm structure.[94] Such observations suggest that there may be opportunity to improve the delivery and efficacy of antibacterial agents in general, through the effects of L-arginine on biofilm architecture.

Based on evolving research centered around L-arginine and the role it plays in the oral cavity by influencing biological processes, modulating the biofilm, and interacting directly with hard tissue surfaces, it is likely that L-arginine possesses further unique, untapped potential. Combining L-arginine with antibacterial actives like zinc, could help to overcome barriers to delivery, biofilm penetration, and retention of zinc through multiple mechanisms of action, including biofilm destabilization and active complexation of zinc to deliver optimized antibacterial efficacy.

Creating a Tailored Solution to Change the Paradigm of Prevention and Oral Health

Clinical research has provided abundant evidence that dental caries and periodontal disease are preventable, and there is recent consensus among academic experts that effective biofilm management is a key strategy for their prevention. However, data on the current prevalence of these diseases on a global basis clearly indicate that routine mechanical oral hygiene measures are insufficient for most individuals to achieve effective disease prevention and good oral health. For this reason, experts also recently agreed that supplementing routine oral hygiene procedures with an adjunctive therapy, that combines fluoride and an antibacterial agent for simultaneous caries and periodontal disease prevention, can improve oral health outcomes for patients individually, and for the population as a whole. The role of dental professionals in ensuring the success of prevention and supporting the patient to achieve better oral health is now universally recognized. It is, therefore, both timely and appropriate to think beyond traditional methods to a more holistic approach to achieve effective prevention and better oral health.

The Colgate-Palmolive Company has embarked on a journey to change the paradigm of daily prevention. In seeking a truly innovative approach to support this move, Colgate has leveraged the recent change in focus of the dental profession from treatment of disease to active prevention, harnessed the trend to more health-conscious

and educated patients and consumers, built upon new understanding of oral biology and oral health, and exploited advances in technical innovation. In so doing, Colgate has developed and validated a new "holistic" toothpaste that meets today's patient and consumer needs and, at the same time, embraces modern concepts and contemporary approaches to prevention. This represents a truly innovative solution to support the change in the paradigm of prevention. To achieve success, however, it is also important to pursue a contemporary approach to professional-patient communication and patient education that follows the principles of patient-centered care by embracing the needs and expectations of patients, and applying behavioral interventions with positive reinforcement to enhance patient motivation and compliance with prevention for improvements in oral health. Colgate recognizes this need to provide support to dental professionals as they empower their patients to take charge of their daily self-care routines towards achieving whole mouth health.

Colgate is introducing an innovative fluoride toothpaste with a unique Dual Zinc plus Arginine therapeutic agent that enhances delivery, retention, and bioactivity of zinc on soft tissue surfaces and deep into existing oral biofilm. This zinc modulates the oral biofilm through a combination of subtle antibacterial mechanisms (inhibition of bacterial adhesion, inhibition of nutrient uptake, and bacterial metabolism) to help maintain its natural balance, retain the symbiotic relationship with the host, and, at the same time, protect and reinforce the host defense mechanisms within the soft tissues. In addition to the other provided benefits, the toothpaste offers patients and consumers "whole mouth protection" against future detrimental oral challenges and "whole mouth health," with only a simple change in oral hygiene regimen. From a population perspective, this toothpaste can play an important role for consumers. With meaningful messaging that motivates and empowers a consumer to improve his/her oral hygiene procedures (self-care), this toothpaste can help enhance success in prevention and improve oral health for everyone. From a professional point of view, this toothpaste can play an important role in a preventive program designed for an individual patient by a dentist or hygienist that empowers the patient to enhance his or her chance of success in prevention and achievement of good oral health. Other components of such an individualized program might include regular professional tooth cleaning, personal oral hygiene instruction enriched by motivational approaches to improve plaque control, and education, coaching, and motivation to reduce specific behavioral risk factors, such as dietary sugar intake and smoking. Furthermore, this toothpaste will continue to have a place in the regimen of healthy patients who will want to benefit from ongoing whole mouth protection to maintain their good oral health status. Thus, it may provide a new standard of care in prevention.

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Enhanced *In Vitro* Zinc Bioavailability through Rational Design of a Dual Zinc plus Arginine Dentifrice

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Abstract

- **Objective:** To investigate bioavailability enhancement of zinc on model oral surfaces and in oral biofilms *in vitro* through strategic formulation with two sources of zinc and L-arginine.
- **Methods:** To modulate the bioavailability of active zinc ions in a zinc citrate dentifrice, an additive research strategy was pursued. A series of zinc citrate dentifrice formulations were prepared with increasing replacement of zinc citrate with zinc oxide (a water insoluble source of zinc ions) to generate a Dual Zinc active system. A screening of isolated zinc and amino acid effects in simple solutions using zeta potential and uptake to model oral surfaces was performed in an effort to determine the effect of particle charge on zinc bioavailability. Zinc delivery and antibacterial efficacy of the Dual Zinc plus Arginine dentifrice formula were tested using *in vitro* oral epithelial tissue and saliva-derived biofilm models. Furthermore, zinc penetration and retention were determined by subjecting *in vitro* biofilms to dynamic flow after treatment with the Dual Zinc plus Arginine dentifrice with treated biofilms evaluated for zinc using imaging mass spectrometry (I-MS). Bacterial adhesion to gingival epithelial cells treated with the Dual Zinc plus Arginine dentifrice was imaged upon challenging with *Streptococcus gordonii*.
- **Results:** Addition of zinc oxide into a zinc citrate dentifrice formula enhanced the efficacy of the system against anaerobic biofilms in a concentration-dependent manner. L-arginine further provided a significant positive charge (+36 mV) to the zinc oxide suspension (+16 mV) as measured by zeta potential. Simple solutions of the Dual Zinc active showed increased zinc uptake on model oral surfaces as a direct function of L-arginine concentration. Antibacterial efficacy of a Dual Zinc plus Arginine dentifrice was evaluated through multiple mechanisms. Enhanced antibacterial performance was observed through significant reductions in metabolic activity as measured through bacterial glycolytic function ($p \leq 0.0001$) and total oxygen consumption ($p \leq 0.0001$). Greater penetration and retention of zinc was observed in bacterial biofilms treated with the Dual Zinc plus Arginine dentifrice in comparison to treatment with a Dual Zinc dentifrice after twelve hours of dynamic flow (10 mL/hour) in an *in vitro* drip flow biofilm culture. Confocal microscopy showed adherent bacteria on cheek cells treated with the Dual Zinc plus Arginine dentifrice formula.
- **Conclusion:** The combination of zinc citrate, zinc oxide, and the amino acid L-arginine in a dentifrice formula enhances the bioavailability of zinc to model oral tissue surfaces, resulting in unique physicochemical effects. The significant antimicrobial control associated with the Dual Zinc plus Arginine dentifrice provides a unique vehicle toward achieving whole mouth health.

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Introduction

Delivery of active ingredients (antibacterials, fluorides, and whitening agents) in a dentifrice base has historically proven to be an effective means in the modulation of oral health. However, formulation design can be complex. The efficacy of an active ingredient against an intended target must be maximized via release from the product matrix during brushing. Furthermore, an active agent must be substantive. The agent should be retained on oral structures at a threshold significant enough to provide protection between product exposures. Simultaneously, the product must provide this efficacy while promoting patient compliance driven through superior product esthetics.

Beyond the design constraints outlined above, progressive assessment into the role of the oral microbiome in health and disease has shifted treatment strategies against oral bacteria from reactive to proactive approaches.[1] The oral microbial community is composed of more than 700 bacterial species coexisting interdependently influencing an individual's oral health status.[1-3] Bacteria colonize on oral surfaces proportional to anatomical surface area with the majority found on soft tissue surfaces (cheeks, gums, and tongue).[4] Oral microflora can impart benefits to the host, including immunomodulation.[5] However, dysbiosis through the colonization of pathogenic bacteria can alter this healthy, balanced environment leading to a diseased state.[6] For example, increased microbial burden along the gingival margin, along with an influx of pathogenic bacteria, are associated with gingival inflammation and gum disease. Antibacterial oral care products should, therefore, focus on controlling the oral bacterial load to reduce the risk of oral disease without depleting the benefits of a healthy oral microflora community.

Interventional oral bacterial control of this kind can be difficult to achieve given the intrinsic protection offered by the biofilm architecture.[7-9] Biofilms are coated with exopolymeric substances (EPS) comprised of proteins, nucleic acids, and polysaccharides; these components collectively serve as a protective barrier against immunologic attack and antibacterial-mediated clearance.[8,10-14] Strategies through chemical or mechanical means must be sought to aid in the delivery,

penetration, and substantivity of actives to biofilms for optimal efficacy. A combination of a bacteriostatic active and an agent to enhance the delivery of that agent to biofilms may provide an interesting approach to bacterial control.

Zinc has a history of benefits in oral health, largely centered around the unique effects of zinc chemistry on oral biology. Application of zinc-based mouthwashes and toothpastes has shown significant reductions in dental plaque.[15-17] Decreases in oral malodor through bacterial control and reaction with volatile sulfur compounds are also well documented in the field.[18] The chemistry of zinc further reduces calculus through bacterial control and inhibition of calcium phosphate crystallization in dental plaque.[19-21] Zinc action is rooted in controlling bacterial growth and subsequent biofilm formation.[22-28] The metal ion has been observed to possess antibacterial function through targeting various bacterial metabolic pathways, resulting in microbial stress and eventually leading to the loss of bacterial viability.[29-39] Additionally, zinc treatment has been shown to significantly impact bacterial accumulation, leading to biofilm reduction over time.[15,29,40,41] Furthermore, a number of studies have shown that the zinc cation can inhibit bacterial adherence to epithelial tissue surfaces, thereby improving gingival tissue barrier integrity.[42]

The efficacy of zinc in the modulation of oral health is attributed to several factors; namely, the complexation and speciation chemistry of zinc,[43-46] its ability to penetrate the biofilm,[47] and its retention and clearance in the oral cavity. A rational design to deliver zinc in an efficacious manner in the oral cavity must take all these factors into consideration. Historically, oral care products have solely utilized water soluble zinc ion sources, such as zinc citrate, zinc lactate, or zinc gluconate. A potential pitfall of this approach is that soluble sources of zinc may be rapidly cleared from the oral cavity. Furthermore, penetration of zinc ions within the biofilm can be limited by the diffusion barrier of the biofilm architecture obstructing bacterial control.[48] Given the landscape, this suggests there is room for improvement in the intraoral delivery of zinc ions from a dentifrice.

The use of slightly soluble sources of zinc in conjunction with soluble sources of zinc presents a unique avenue to control the delivery and release of zinc in the oral cavity. The former may serve as a slow release source of zinc ions and the latter as a readily available form of zinc ions. Zinc oxide, which is considered insoluble in water, could be a source of zinc ions in the mouth by being solubilized and broken down through exposure to chelates of a higher binding constant in an aqueous environment at physiological pH (like saliva, biofilms, or oral structures), if it is retained for sufficient time in the oral cavity.

To optimally leverage the unique release characteristics of a mixed or Dual Zinc system, measures must be taken to ensure penetration of bacterial biofilms and intraoral retention. L-arginine is a semi-essential amino acid that is unique from all other amino acids. L-arginine carries a net positive charge at physiological pH via a distinct guanidinium-based side chain that is involved in numerous biological processes.[49] L-arginine has been used in a number of oral care products to provide enhanced hard tissue benefits. It has both a physicochemical and biological mode of action. The physicochemical mode of action has been leveraged to develop highly effective dentifrices for treating dentin sensitivity. In these dentifrices, L-arginine is combined with an insoluble source of calcium. This interaction allows the calcium to adhere to the dentin surface and serve as a physical plug to occlude open dentin tubules, which are the source of dentin hypersensitivity.[50,51] L-arginine has also been used in fluoride dentifrices containing calcium abrasives to significantly improve anticaries efficacy through promotion of a more alkaline environment that can limit cariogenic microbes from thriving.[52,53]

While the use of L-arginine in oral care products has largely been focused on conditions afflicting the hard tissue, L-arginine has also been shown to enhance the antibacterial performance of actives against bacteria by improving active penetration into biofilms.[54,55] While the exact mechanism by which L-arginine works in combination with antibacterial actives remains unknown, studies by Kolderman, *et al*., and He, *et al*. suggest that the amino acid affects biofilm integrity through the disruption, as well as inhibition of EPS formation.[55-57] Further studies have shown improved efficacy of an antibacterial agent against bacteria in biofilms via destabilization of the biofilm architecture following L-arginine exposure.[54-56] These studies collectively suggest that these unique properties of L-arginine may be leveraged as a potential mechanism to enhance the uptake and retention of zinc into biofilms.

The Colgate-Palmolive Company (New York, NY, USA) has developed a silica-based fluoride toothpaste that utilizes the unique combination of two forms of zinc, zinc citrate and zinc oxide, plus L-arginine to enhance the delivery, retention, and release of the active zinc system in the oral cavity. The objective of this paper is to investigate the enhanced bioavailability to oral structures and biofilm control properties of a Dual Zinc (zinc citrate and zinc oxide) plus Arginine active system delivered through a dentifrice *in vitro*. This progressive strategy toward enhancing zinc bioavailability centered on both potential modulation of zinc ion release via complexes with two different zinc species with different solubility profiles, and the use of the L-arginine to enhance the uptake, penetration, and retention of zinc ions in bacterial biofilms. The physicochemical and biological effects of these strategic approaches on model oral surfaces and plaque biofilms were evaluated to provide a perspective on the potential of these active combinations to control dental plaque and promote oral health.

Materials and Methods

Zinc oxide (ZnO) was purchased from U.S. Zinc (Houston, TX, USA). Zinc citrate trihydrate was supplied through Jost Chemical (St. Louis, MO, USA). All amino acids were purchased from Ajinomoto (Tokyo, Japan). The pH of the prepared simple solutions was adjusted with sodium hydroxide (NaOH) or hydrochloric acid (HCl) as noted. Hydroxyapatite (HAP) disks (0.5" x 0.04–0.06") were purchased from Himed (Old Bethpage, NY, USA). Vitro Skin® was purchased from IMS Testing Group (Portland, ME, USA) and used as directed by the manufacturer.

A dentifrice containing 0.96% zinc (zinc oxide, zinc citrate), 1.5% L-arginine, and 1450 ppm fluoride as sodium fluoride in a silica base (Colgate-Palmolive Co., New York, NY, USA) and a dentifrice containing 0.96% zinc (zinc oxide, zinc citrate) and 1450 ppm fluoride as sodium fluoride in a silica base (Colgate-Palmolive Co., New York, NY, USA) were used throughout the course of this study. Human saliva and gingival epithelial cells were collected from volunteer donors following approval by the local ethics approval board.

Zinc concentration as noted throughout different studies on model oral substrates and biological specimens, and bacterial biofilms were

determined via inductively coupled plasma-atomic emission spectroscopy (ICP-AES) on an Agilent ICP-OES 5100 SVDV ICP-OES spectrophotometer (Agilent, Santa Clara, CA, USA) using the following measurement conditions: 10-second read time, nebulizer flow rate 0.65 L/min, RF power 1.3 kW, plasma flow 12.0 L/min, stabilization time 15 s, aux flow 1.0 L/min.

Anaerobic Biofilm

The effect of the test dentifrices on bacterial growth was tested in an anaerobic biofilm model. Whole saliva was harvested from a total of four volunteers and pooled for a single inoculum. The OD of the inoculum was adjusted to an absorbance of approximately 0.3 (610 nm). Sterile HAP disks were incubated for 24 hours at 37°C under anaerobic conditions in sterile artificial saliva containing 0.01% sucrose (1 mL) and pooled saliva (1 mL) in a 24-well plate.

Disks were treated with a 1:2 (w/w) slurry of diluted dentifrice in water for 10 minutes and then transferred into sterile artificial saliva (2 mL). Disks were treated once per day for a total of eight days. At days two, four, and eight, the disks were collected and transferred to 0.5x pre-reduced thioglycollate medium. Samples were diluted and plated on Neomycin-Vancomycin (NV) agar to quantify total Gram-negative anaerobes. Plates were incubated anaerobically at 37°C for 72 hours before determining total colony counts. Results are reported as log (CFU/mL) for triplicate samples.

Zeta Potential

The effect on zinc oxide particle charge upon exposure to amino acids was screened using zeta potential. Specific amino acids were selected based on side chain functionality: L-serine (polar, neutral), L-arginine (polar, cationic), and L-glutamic acid (polar, anionic). For zeta potential measurements, select amino acids (1.7 mmol) were added to aqueous suspensions of zinc oxide (12 mM). This concentration of zinc oxide was studied so as to minimize aggregation during zeta potential measurements. The pH of each system was adjusted to approximately pH 8. Each amino acid-zinc oxide solution was vortexed, sonicated, and then loaded into a Zetasizer DTS 1061 capillary cuvette. The cuvette was placed in the Zetasizer instrument and 12 zeta runs were performed. An average zeta potential value was calculated from the results.

HAP Disk Uptake

To determine the effect of L-arginine on Dual Zinc in simple systems, a series of aqueous solutions of zinc citrate, zinc oxide, and L-arginine were prepared. The solids of each solution were dispersed in deionized water and followed by adjustment to pH 7.0 (±0.15) brought to a total volume of 500 mL. Zinc concentration was held constant at 100 mM through a combination of zinc citrate trihydrate (1.6 g, 2.5 mmol) and zinc oxide (3.5 g, 42.5 mmol). Three solutions were prepared by addition of L-arginine at three different levels (1.6 g, 9.2 mmol, 5.2 g, 30 mmol, and 10.5 g, 60 mmol).

HAP disks were transferred to a 24-well plate (one disk per well). Parafilm-stimulated saliva was collected from a volunteer donor, centrifuged at 8000 rpm for 10 minutes, and the supernatant filter sterilized by passing through a 0.45 µm vacuum filtration device. A portion of the filtered, sterile salivary supernatant (1 mL) was added to each well. The plate was incubated at 37°C for one hour, allowing for pellicle formation.

Saliva was removed and the HAP disks were incubated with the soluble fraction of each simple solution (1 mL) for two minutes. For toothpaste samples, HAP disks were treated with 1:2 (w/w) toothpaste:deionized water slurries. Samples were performed in triplicate. Samples were aspirated and deionized water (1 mL) added to wash each HAP disk. This process was repeated twice to provide a HAP disk free of nonspecifically bound ingredients. Concentrated nitric acid (0.5 mL, 70%) was used to digest the treated HAP disk. Upon complete dissolution of the material, samples were diluted with deionized water (4.5 mL to a total volume of 5.0 mL) for quantitative analysis by ICP-OES.

Vitro Skin Deposition

A punch device was used to cut Vitro Skin from bulk sheets into disks 7 mm in diameter. Vitro Skin disks were hydrated overnight in a hydration chamber (IMS Testing Group) over a 15:85 glycerin (44 g):deionized water (256 g) solution. The Vitro Skin disks were then transferred to a 24-well plate (one disk per well). Parafilm-stimulated saliva was collected and centrifuged at 8000 rpm for 10 minutes. A portion of the salivary supernatant (1 mL) was added to each well. The plate was incubated at 37°C for two hours on an orbital shaker, rotating at 110 rpm to allow for pellicle formation.

The Vitro Skin disks were incubated with an aliquot of the soluble fraction of each simple solution (1 mL) for two minutes. Samples of each simple solution were performed in triplicate. The simple solutions were aspirated and deionized water (1 mL) added to wash each Vitro Skin disk. This process was repeated twice to provide a Vitro Skin free of nonspecifically bound ingredients. Concentrated nitric acid (0.5 mL, 70%) was used to digest the sample. Upon complete dissolution of the material, samples were diluted with deionized water (4.5 mL to a total volume of 5.0 mL) for quantitative analysis by ICP-OES.

Zinc Deposition on MatTek, Epigingival™ Tissue

MatTek Epigingival™ tissues (GIN-606, Ashland, MA, USA) were treated with diluted dentifrice slurry [1 mL/tissue, 1:2 in deionized water (w/w)] for two minutes at room temperature. Tissues were washed with phosphate-buffered saline (PBS, 2 mL) three times and transferred into fresh tubes, one tissue per tube. Tissues were digested with nitric acid (70%, 0.5 mL) at room temperature overnight. Digested samples were diluted with deionized water (4.5 mL to a total volume of 5.0 mL), followed by centrifugation of the tubes at 4000 rpm for ten minutes. The supernatant of each sample was transferred into a fresh tube for analysis with ICP-OES.

Zinc Deposition in Biofilms

To determine the amount of zinc delivered to biofilms as a function of dentifrice product, salivary biofilms were grown on vertically suspended HAP disks for 48 hours at 37°C under a 5% CO_2 environment. Biofilms were cultured in McBain medium [2.0 g/L BactoPeptone (Difco, Detroit, MI, USA), 2.0 g/L Trypticase Peptone (BD, Franklin Lakes, NJ. USA), 1.0 g/L yeast extract (BD), 0.35 g/L sodium chloride (Sigma-Aldrich, St. Louis, MO, USA), 0.2 g/L potassium chloride, 0.2 g/L calcium chloride, 2.5 g/L mucin, and 50 mmol/L PIPES, (pH = 7.0)] supplemented with 5 µg/mL hemin and 1 µg/mL menadione. The medium was refreshed a total of four times at approximately

12-hour intervals. Each biofilm was then treated once with an aliquot of dentifrice slurry diluted in sterile deionized water [1.5 mL, 1:2 (w/w)] for two minutes. The dentifrice slurry was aspirated and the biofilm washed twice in sterile deionized water for five minutes. The treated biofilms were transferred into sterile deionized water (700 µL) by sonication using a Virtis virsonic 600 (80% power for two minutes per disk side at 30-second intervals).

Nitric acid (0.5 mL, 70%) was added to each treated biofilm sample and left to digest overnight. Upon complete dissolution of the material, samples were diluted with deionized water (to a total volume of 5.0 mL) for quantitative analysis by ICP-OES. A total of three experimental replicates with six individual biofilms per dentifrice treatment were analyzed. A different saliva donor was used to derive the biofilm per experimental replicate.

Microbial Metabolic Function

The effect of the test dentifrices on bacterial metabolic function was evaluated through measurement of bacterial respiration and extracellular acidification rates. Multispecies oral biofilms from an unbrushed saliva inoculum were cultured vertically on HAP disks in McBain media supplemented with 5 µg/mL hemin, 1 µg/mL menadione, and 0.2% sucrose at 37°C for 48 hours under an environment containing 5% CO_2. Resulting biofilms were harvested in water by vigorous pipetting. The dislodged bacteria were reconstituted into fresh 0.25X media [tryptic soy broth (TSB) + 0.2% sucrose], and the bacterial suspension adjusted to a final optical density (OD) of approximately 0.7 (610 nm). An aliquot of the diluted bacterial suspension (10 µL), the diluted toothpaste slurry [12 µL, 1:10, (w/w)], and media (180 µL) were added to XF Cell Culture Microplates precoated with Corning Cell Tak. The resulting reaction mixture was then centrifuged for 10 minutes at 1500x g at room temperature. Real-time oxygen consumption rates (OCR) and extracellular acidification rates (ECAR) for multi-species bacteria derived from biofilms were determined using the Seahorse Extracellular Flux (XF24) analyzer (Seahorse Bioscience, MA, USA). The microplate was loaded to the analyzer measuring changes in OCR and ECAR over 50 cycles (4.5 hours) in response to treatment. The area under the curve (AUC) was calculated for all 50 cycles upon completion of the assay using SciDavis software. Experimental replicates corresponded to biofilms derived from new saliva donors.

Aerobic Biofilm

The effect of the test dentifrices on bacterial growth was tested in an aerobic biofilm model. Whole saliva was pooled from three volunteers and centrifuged for 10 minutes at 8000 rpm. The supernatant was collected and sterilized by UV light and filtered. An aliquot of sterilized human salivary supernatant (1.5 mL) was transferred to each well of a 24-well sterile culture plate. HAP disks held in a vertical position by a modified steel lid were suspended in the saliva and incubated for one hour at 37°C to allow a pellicle to form.

Aliquots of diluted dentifrice slurry in deionized water [1.5 mL, 1:3 (w/w)] were placed in the appropriate wells of a sterile 24-well plate. Pellicle-coated disks were transferred to this plate and incubated for two minutes at room temperature with vigorous shaking on an orbital shaker. Following treatment, the HAP disks were rinsed two times for five minutes each in a plate containing fresh, sterile 0.25X TSB (1.5 mL/well) with the same vigorous shaking. HAP disks were then transferred to a plate containing SHI medium (Teknova) with 25% whole saliva from a single donor and incubated (37°C, 5% CO_2) for four hours to allow for initial colonization to occur. Following incubation, a second treatment was performed in the same manner as previously described. HAP disks were transferred to a plate containing sterile SHI medium with no further inoculum applied to the experiment. For four subsequent days, the plates were removed at 24-hour intervals from the initial treatment and treated again, as above.

Following the sixth and final treatment, the disks were incubated for an additional two to three hours to allow the bacteria to recover. Disks were then transferred to individual 15 mL round bottom test tubes containing 0.25% trypsin solution in water (2 mL). HAP disks were incubated in trypsin at 37ºC for one hour to remove the biofilm from the disks. Following trypsinization, biofilm bacteria were quantified for viability remaining after treatment. Bacteria samples were diluted and plated on blood agar to quantify for total aerobic bacteria. Plates were incubated aerobically at 37°C for 24–48 hours before determining total colony counts. Results are reported as log (CFU/mL) for triplicate samples.

Metal Penetration and Retention Assays

Zinc penetration and retention in salivary biofilms were evaluated using a laboratory model with a continuous media flow. Sterile HAP-coated glass microscope slides were pre-incubated with individually collected saliva inoculum containing saliva and plaque-derived bacteria for two hours at 37ºC under an environment containing 5% CO_2. The inoculated slides were then transferred into a drip-flow biofilm reactor (Biosurface Technologies Corporation, Bozeman, MT, USA) and incubated at 37ºC. The biofilms were cultured under a constant flow rate of 10 mL/hour of growth medium consisting of 0.55 g/L proteose peptone (BD), 0.29 g/L trypticase peptone, 0.15 g/L potassium chloride (Sigma-Aldrich, St. Louis, MO, USA), 0.029 g/L cysteine-HCL, 0.29 g/L yeast extract, 1.46 g/L dextrose, and 0.72 g/L mucin. The medium was supplemented with sodium lactate (0.024%, final concentration) and hemin (0.0016 mg/mL, final concentration). The biofilms were cultured for a total of 10 days. The resulting biofilms were then treated with dentifrice slurry diluted in sterile deionized water [1:2 (w/w)] for two minutes. Following treatment, the biofilms were washed twice in sterile deionized water (five-minute intervals) and then placed back into the biofilm reactors, resuming biofilm culture as previously described. The treated biofilms were allowed to recover for approximately 12 hours. The resultant biofilms were harvested by flash-freezing in liquid nitrogen and excised from the glass slides while carefully maintaining their orientation.

The biofilms were stored at -80ºC until analyzed by imaging mass spectroscopy. Biofilm samples were analyzed by Protea Biosciences (Morgantown, WV, USA) using Bruker UltrafleXtreme MALDI TOF/TOF. The biofilms were cryosectioned at 16 µm thickness and placed on stainless steel MALDI targets. The biofilms were coated with sinapinic acid (10 mg/mL, at a flow rate of 30 µL/min for a total of 30 coats) and allowed to dry for 20 seconds prior to analysis. The biofilm samples were ablated at 200 laser shots per pixel at a spatial resolution of 50 µm using reflectron positive ion mode. Sample mass ranges of between 100-1000 Daltons were collected and the images visualized using Bruker Flex Imaging.

Bacterial Challenge Assay

The effect of the test dentifrice treatment in limiting bacterial adhesion was determined *in vitro* on gingival epithelial cells. Gingival epithelial cells were collected from three volunteer donors using a sterile cotton swab with gentle scraping along the gum area. The collected cells were resuspended in sterile PBS (4 mL) and enriched via centrifugation at 8000 rpm for ten minutes. The resulting cellular pellet was resuspended in PBS (400 µL). The isolated gingival epithelial cells were treated with diluted dentifrice slurry [5 µL, 1:10 in water (w/w)] for approximately two minutes. The treated cells were collected via centrifugation at 8000 rpm for 10 minutes and resuspended in Hanks Balanced Salt Solution (HBSS, 1 mL). The resulting cells were then challenged as described below with *Streptococcus gordonii* DL-1 endogenously expressing mCherry (created as described by Aspiras, *et al*.[58]). *S. gordonii* were cultured in Brain Heart Infusion broth supplemented with erythromycin [5 µg/mL, (final concentration)] and cultured at 37°C under 5% CO_2 environment for 48 hours. Prior to challenge, the bacterial culture was resuspended separately in HBSS to a final optical density of 0.1 (610 nm). An aliquot of the bacterial suspension (100 µL) was then added to the treated epithelial cells and co-incubated in a 37°C orbital shaker for two hours at 80 rpm. Non-adherent cells were removed by centrifugation at 1000 rpm for five minutes and the cell pellet resuspended in HBSS. The cells were washed a total of three times. Following the wash steps, the cell pellet was resuspended in ProLong Gold DAPI (100 µL), and mounted on glass slides. The samples were visualized by confocal microscopy using Nikon C2siR (Melville, NY, USA) under 40X magnification. The samples were imaged using solid state lasers at 405 nm and 561 nm to detect DAPI and mCherry. DiC images were collected using a 488nm laser. Z-plane scans from 0-30 µm were collected with a total of three to four randomly chosen z-stack images per treatment per volunteer sample (n =3).

Results

Dentifrice prototypes were prepared in which the amounts of zinc oxide to zinc citrate were varied while holding the total zinc concentration constant at approximately 1% (w/w) in the formulation. The antibacterial efficacy of these dentifrice formulations was tested in an anaerobic biofilm model. The presence of insoluble and soluble sources of zinc in a dentifrice was shown to decrease bacterial viability in comparison to a placebo control (Figure 1). Furthermore, as the ratio of insoluble to soluble sources of zinc increased, the antibacterial efficacy of the formula was seen to increase. Sequential reductions in the total Gram negative bacteria were observed with an increasing ratio of zinc oxide, even though the total zinc levels were kept the same in all formulas.

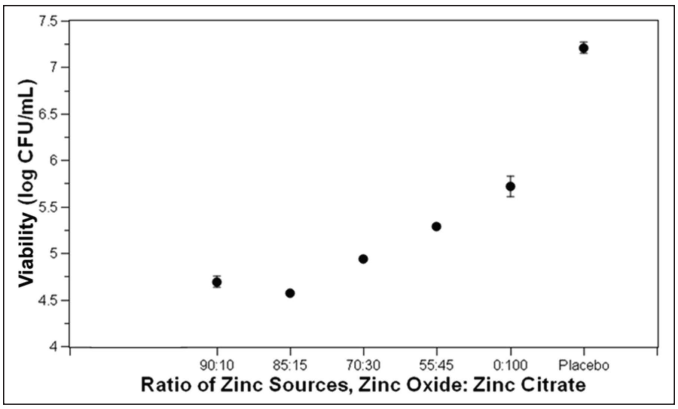

Figure 1. *Antibacterial efficacy (reported as total Gram negative anaerobes on NV agar) of dentifrice formulas containing different ratios of zinc oxide and zinc citrate with the total zinc level in the formula held constant.*

Given the propensity for further modulation of zinc charge in relation to bioavailability, a screening of zinc oxide interactions with amino acids was pursued. Specific amino acids were selected based on side chain functionality: L-serine (polar, neutral), L-arginine (polar, cationic), and L-glutamic acid (polar, anionic). To differentiate amino acid effects on zinc charge, zeta potential was used to determine the charge of zinc oxide in the presence of each amino acid (Table I). Zinc oxide alone carries a net positive surface charge at pH 8 (+16 mV). Addition of L-serine did not alter the charge, while L-glutamic acid altered zinc oxide to a net negative charge (-28 mV). Supplementation of L-arginine was shown to generate a large positive charge in solution in comparison to the other amino acids tested (+36 mV).

Table I
Zeta Potential (mV) in Zinc Oxide and L-Amino Acid Simple Solutions at pH 8

Amino Acid	ZnO Only	ZnO Only + L-Arginine	ZnO Only + L-Serine	ZnO Only + L-Glutamic Acid
Zeta Potential (mV)	+16	+36	+16	-28

Based on the strong positive charge of this interaction, simple aqueous solution combinations of zinc oxide and zinc citrate plus L-arginine were pursued to evaluate zinc deposition propensity on model oral surfaces. Dual Zinc mixtures (zinc oxide and zinc citrate, 85:15 ratio) were supplemented with increasing concentrations of L-arginine (20 mM, 60 mM, and 120 mM). When model oral surfaces were exposed to the soluble phase of each aqueous suspension, zinc uptake was shown to increase proportionally to the amount of L-arginine (Figure 2).

Dentifrice prototypes containing Dual Zinc (zinc citrate and zinc oxide) with or without L-arginine were designed. These formulas were evaluated against a regular fluoride toothpaste for zinc deposition in an EpiGingival tissue model comprised of oral epithelial cells of human origin and in static human saliva-derived bacterial biofilms [not subjected to flow, (Figure 3)]. Treatment with the Dual Zinc or the Dual Zinc plus Arginine dentifrice slurry deposited significant amounts of zinc in comparison to a non-metal-containing regular fluoride toothpaste in both the oral models. Although both prototypes were formulated at equal molar concentrations of zinc, the role of L-arginine in zinc delivery was observed through statistically significant increases in zinc deposition to the oral epithelial surface model (26.5%; $p = 0.0157$) and the treated bacterial biofilms (25%; $p \leq 0.00001$) when compared against model-respective samples treated with Dual Zinc technology alone.

Given the diverse modes of action of zinc against bacteria, multiple *in vitro* models were pursued to elucidate the extent to which the enhanced bioavailability of the Dual Zinc plus Arginine dentifrice impacted bacterial control. The capability of the product to disrupt bacterial metabolic functions was first assessed through impacts on the glycolytic and respiratory pathways of bacterial biofilms. The results of this assessment are found in Table II. Biofilms treated with either zinc-based toothpaste resulted in significant reductions in respiration and metabolic activity of the bacteria as measured by OCR

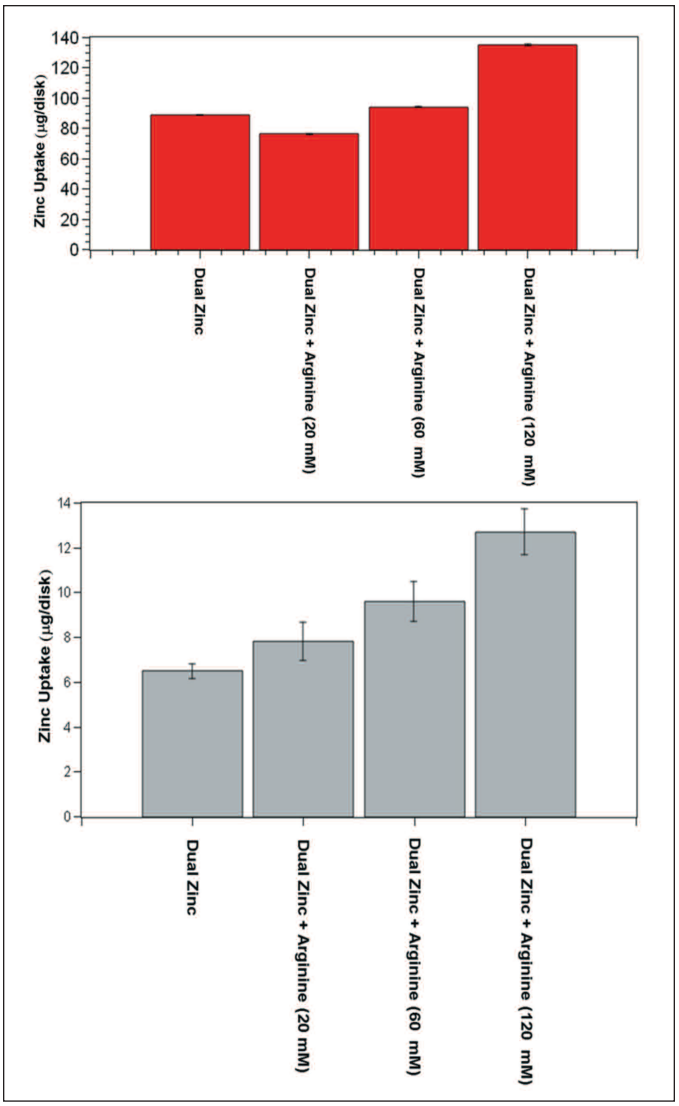

Figure 2. *Zinc uptake from Dual Zinc aqueous solutions to synthetic oral surfaces as a function of L-arginine concentration. [HAP (top) and Vitro Skin (bottom)].*

Table II
Rate Comparisons in Bacterial Metabolic Function Post-Toothpaste Treatment

Toothpaste Used for Treatment	[a]Oxygen Consumption Rate ± Standard Deviation (pmol/min)	[b]Extracellular Acidification Rate ± Standard Deviation (mpH/min)
Untreated	66.1796 ± 7.64	11.9568 ± 1.2928
Non-Antimicrobial Fluoride Toothpaste	67.2654 ± 4.2067	10.745 ± 1.2614
Zinc Citrate	12.5635 ± 1.5334	2.6482 ± 0.3417
Dual Zinc	19.9314 ± 1.1079	4.2789 ± 0.6013
Dual Zinc + Arginine	0.87147 ± 3.218[c]	0.1001 ± 0.2955[d]

[a]Average OCR was calculated after the first 10 cycles (80 minutes) post toothpaste treatment.
[b]Average ECAR was calculated after the first 7 cycles (56 minutes) post toothpaste treatment.
[c]Indicated significant reduction in OCR vs. Dual Zinc ($p \leq 0.0002$) treated bacterial samples.
[d]Indicates significant reduction in ECAT vs. Dual Zinc ($p \leq 0.0001$) treated bacterial samples.

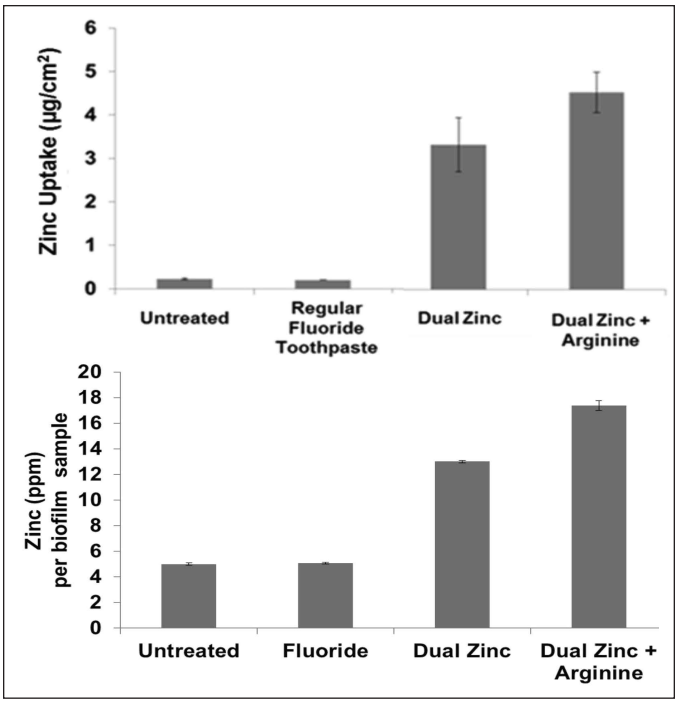

Figure 3. *Zinc uptake to the EpiGingival tissue model consisting of oral epithelial cells of human origin (top) and to bacterial biofilms (bottom) upon exposure to a 1:2 dentifrice slurries.*

and ECAR in comparison to untreated ($p < 0.00001$) and regular fluoride toothpaste ($p < 0.00001$) controls (Table II). Moreover, bacteria treated with the Dual Zinc plus Arginine dentifrice showed statistically significant ($p < 0.0002$) reductions in respiration and metabolic activity as measured by OCR and ECAR in comparison to treatment with the Dual Zinc-only dentifrice (consuming only 0.9 pmol/min of oxygen). Referring to Figure 4, bacteria exposed to either zinc product consumed significantly less oxygen over the course of 300 minutes in comparison to untreated bacteria and those treated with a regular fluoride toothpaste. Moreover, bacteria treated with the Dual Zinc plus Arginine dentifrice showed statistically significant ($p < 0.0001$) reductions in bacterial respiratory function in comparison to the Dual Zinc-treated bacterial biofilm, indicating that L-arginine is modulating the efficacy of zinc. Quantification of total oxygen consumed based on AUC showed Dual Zinc plus Arginine dentifrice treatment significantly reduced the bacterial respiration, consuming

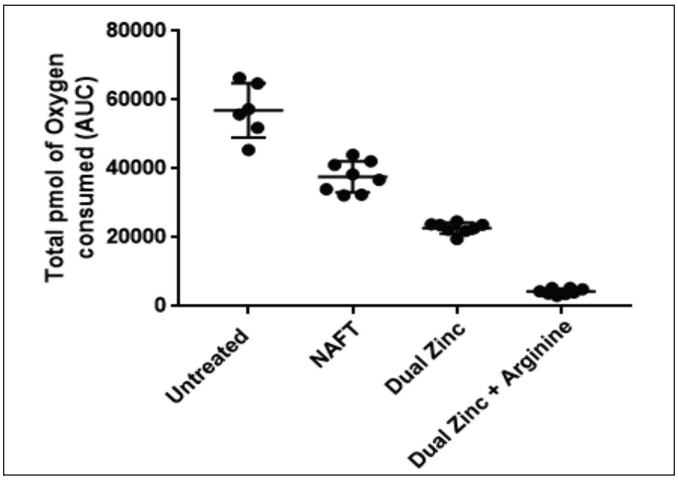

Figure 4. *Comparison of total oxygen consumed by the bacteria based on AUC generated over 300 minutes.*

4301 pmol of oxygen. In comparison, the Dual Zinc dentifrice-treated bacteria still consumed on average 22777 pmol of oxygen.

Treatment with the Dual Zinc plus Arginine dentifrice was further evaluated in aerobic and anaerobic biofilm models for the ability to reduce bacteria viability (Figure 5). Significant reductions (one-way

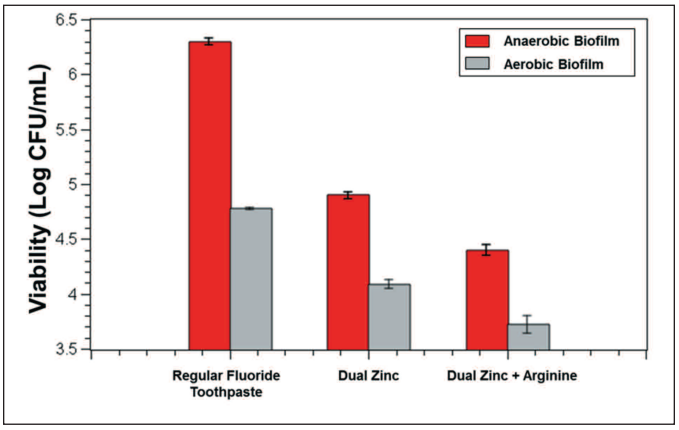

Figure 5. *Reductions in bacterial biofilms viability (log CFU count) under aerobic and anaerobic conditions upon dentifrice treatment.*

ANOVA) in the viability of the bacterial biofilms (as measured by bacterial colony forming units) were observed for treatment with the Dual Zinc and Dual Zinc plus Arginine dentifrices in comparison to treatment with a regular fluoride toothpaste ($p < 0.05$). L-arginine again enhanced the delivery and bioavailability of the zinc cation, with bacterial reductions significantly greater ($p < 0.05$) than the biofilms treated with Dual Zinc-only dentifrice.

The role of L-arginine in the enhanced delivery and bioavailability of zinc in the Dual Zinc plus Arginine dentifrice was further differentiated by evaluating zinc penetration and retention in bacterial biofilms under flow conditions. Imaging MALDI-MS (I-MS) was used to quantify the amount of zinc remaining in treated bacterial biofilms that were submitted to 12 hours of dynamic flow. A concentration map analysis of the MALDI-MS image is shown in Figure 6, which qualitatively demonstrates that biofilms treated with the Dual Zinc plus Arginine dentifrice exhibited greater levels of zinc penetration and retention in comparison to Dual Zinc dentifrice-treated bacterial biofilms. Biofilms treated with the Dual Zinc-only dentifrice did not

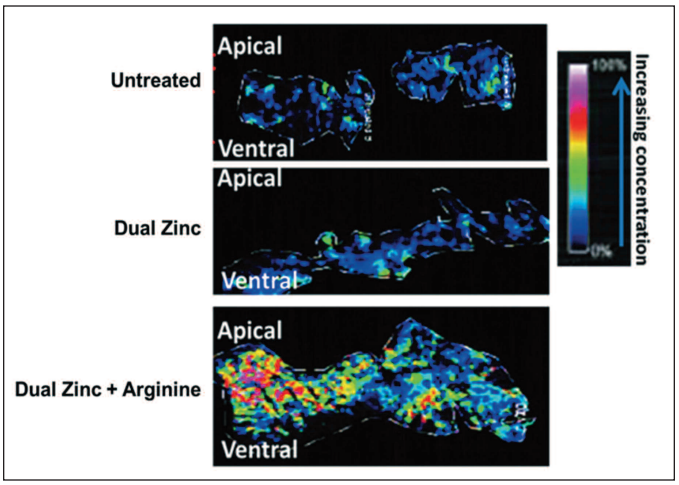

Figure 6. *Zinc visualization using I-MS with heat mapping for zinc in the sagittal section biofilm section in untreated, Dual Zinc dentifrice-treated, and Dual Zinc plus Arginine (DZA) dentifrice-treated biofilms subjected to 12 hours of dynamic flow.*

demonstrate notable retention of the metal when compared to untreated biofilms after 12 hours of dynamic flow, which supports L-arginine's role in the improvement in zinc delivery and retention.

In vitro multimodal assessment of the Dual Zinc plus Arginine dentifrice mechanism of action was also determined through inhibition of bacterial colonization on soft tissue surfaces. Confocal imaging of bacteria-challenged cheek cells treated with the Dual Zinc plus Arginine dentifrice showed less bacteria adherent per gingival cell as compared with cells treated with only a regular fluoride toothpaste (Figure 7). No visual difference was observed between the untreated and regular fluoride-treated cells.

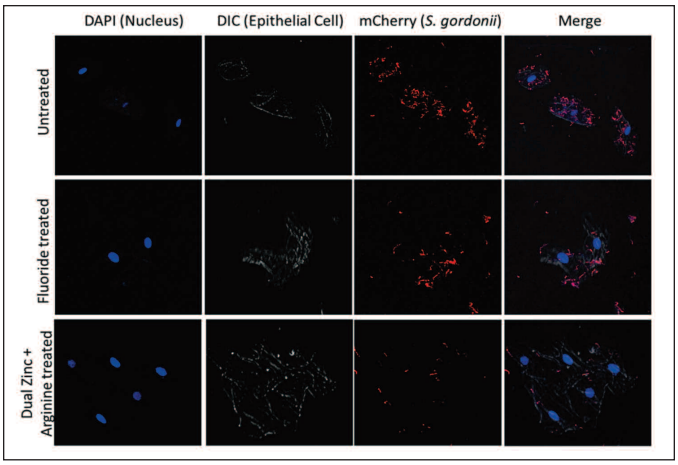

Figure 7. *Confocal imaging of bacteria challenged gingival cells that were treated with the Dual Zinc plus Arginine dentifrice show less adherent bacteria (red) per cell (DiC) as compared with untreated and regular fluoride toothpaste-treated samples.*

Discussion

Zinc delivers antimicrobial and anti-plaque benefits by specifically targeting and reducing bacterial metabolic functions.[23,31,32,39,40,59,60] The efficacy of the zinc cation is contingent upon its bioavailability, with penetration and retention playing a central role in zinc's overall performance against biofilm-associated bacteria. Complexes of zinc, like zinc citrate and zinc lactate, that are adequately soluble in aqueous solutions are effective against a broad spectrum of oral microbes as they provide a large source of readily available zinc ions. However, the antibacterial activity of a zinc source is not controlled by the solubility of the coordination complex, but rather the binding constant of the electron-rich donors coordinating the metal cation. Zinc oxide has minimal solubility in water ($K_{sp} = 11.31$), but a weaker binding constant like zinc citrate ($\log K < 5$).[61] Therefore, zinc oxide is capable of being broken down over time by ligands with greater binding constants. The binding constant of phosphate salts in saliva, sulfur compounds in saliva and breath, and cell membranes of oral tissues and bacteria for zinc is high ($\log K > 5$).[46,62] Chelation of zinc by weaker donors ($\log K < 5$) is advantageous to modulating the oral biological function of zinc cations; oral structures and salts can outcompete the delivery ligand, enhancing zinc activity. In biofilm antibacterial studies, a performance advantage was seen toward having a combination of soluble zinc (zinc citrate) and insoluble/solubilizable zinc source (zinc oxide) likely due to the deposition of both entities. In this case, zinc citrate likely provided initial antibacterial efficacy while zinc oxide acted as a reservoir; large particles presumably deposited in and on the biofilm and were potentially broken down over time, creating a continuous source of zinc ions. An immediate release of

zinc ions (via the more soluble component) would potentially show potent impact upon brushing, while the slow dissolution of zinc particles over time (via the solubilizable/more insoluble component) may provide prolonged protection.

The Dual Zinc (zinc citrate and zinc oxide) active system was further developed through the addition of the amino acid L-arginine to modulate zinc bioavailability and activity within the oral cavity. Modulation of solution equilibria through biologically relevant compounds like amino acids also has the potential to uniquely enhance delivery of zinc ions from a formulation to oral surfaces. Previous studies have shown the capability of ligands like amino acids to enhance the solubility of poorly soluble zinc materials.[63-65] Given the propensity for further modulation of zinc bioavailability, a screening of amino acids with zinc oxide was pursued. Amino acids can interact with zinc oxide, slowly breaking down with the resulting material imparting some chemical properties of the donor. Zeta potential studies showed the charge of the zinc oxide-amino acid mixture was heavily dependent upon the functional group of the amino acid side chain. Oral surfaces (*e.g.,* mucosa and soft tissues) and bacteria have a net negative charge. The high positive charge resulting from interaction with L-arginine under physiological conditions was considered advantageous for targeting these oral surfaces and bacteria-enhancing bioavailability of the zinc dentifrice.

Moreover, L-arginine has been shown to enhance the bioavailability of the Dual Zinc system facilitating deposition, penetration, and retention of zinc in biofilms. Zinc efficacy can be hindered by its ability to effectively penetrate biofilms such as oral plaque, thereby limiting its antimicrobial activity against bacteria. One approach to improve the delivery and efficacy of zinc is through disruption of the biofilm architecture, either through chemical or mechanical means. In an analogous study, L-arginine has been shown to enhance the antibacterial performance of antibiotics against bacteria by improving the penetration of actives within the microbial community, possibly through biofilm destabilization.[54, 55]

The clinical mode of action by the Dual Zinc plus Arginine dentifrice in biofilm control is projected to work against oral bacteria in three ways (Figure 8). Stepwise improvements in zinc deposition on to model oral surfaces were observed with increasing concentrations of L-arginine. *In vitro* analysis has also shown enhanced zinc deposition on both EpiGingival and hydroxyapatite biofilm models upon treatment with the Dual Zinc plus Arginine dentifrice. Despite the biofilms being treated with toothpaste slurries containing equimolar zinc ions, the amount of zinc deposited was significantly greater than treatment with a Dual Zinc-only dentifrice. Furthermore, in bacterial biofilms subjected to dynamic flow, treatment with the Dual Zinc plus Arginine dentifrice imparted significant amounts of zinc, as determined by I-MS studies, in the biofilm even after 12 hours post-treatment. In the same experiment, biofilms treated with a Dual Zinc-only dentifrice showed comparable levels of zinc to regular fluoride toothpaste-treated biofilms. These results suggest that L-arginine in the dentifrice formula may function to enhance the deposition, penetration, and retention of zinc in bacterial biofilms.

Secondly, enhancing the delivery and retention of zinc in biofilms may result in the bacteria being more effectively targeted within those biofilms. Improving the bioavailability of zinc ions in biofilms will, therefore, more effectively inhibit bacterial glycolytic and respiratory function. Results reported in this study are consistent with the known mechanisms of action for zinc[31,66-68] which have been shown to primarily target bacterial metabolism, including vital pathways involved in central carbon metabolism. The bacteriostatic action, quantified in this study by ECAR, can hinder ATP synthesis, leading to bacterial death. This study demonstrated that treatment of bacteria with the Dual Zinc plus Arginine dentifrice led to reduced acidification of the extracellular milieu in comparison to the Dual Zinc-only dentifrice. These data indicate the important role of L-arginine in enhancing zinc function against bacteria through inhibition of bacterial glycolysis.

Similarly, zinc has been shown to impact bacterial oxidative metabolism,[69] thereby reducing oxygen consumption as well as the peroxide neutralization. This study showed that Dual Zinc plus Arginine dentifrice treatment of bacteria led to a significant reduction in OCR in comparison to treatment with the Dual Zinc-only dentifrice. This specific zinc mechanism could have broad ramifications in oral biofilm control as it could lead not only in the inhibition of the growth of oral streptococci[70] and related genera, but could also potentially lead to the clearance of anaerobic periodontal pathogens (*e.g., Fusobacterium nucleatum* and *Porphyromonas gingivalis*) which typically lack protective enzymes against reactive oxygen species.

Finally, these studies demonstrated that treatment with the Dual Zinc plus Arginine dentifrice can influence the absorption of zinc on model oral gingival cells enhancing the barrier defense offered by the oral epithelia against microbial colonization. Confocal imaging studies of Dual Zinc plus Arginine dentifrice-treated gingival cells showed an enhanced resiliency of gingival epithelial cells to bacterial challenge by *Streptococcus gordonii,* a primary bacterial colonizer important in supragingival colonization of periodontal pathogens. Tamura, *et al*. previously illustrated the ability of zinc to interfere with *Porphyromonas gingivalis* colonization of the gingival sulcus by blocking its ability to co-aggregate with the oralis group of streptococci.[70]

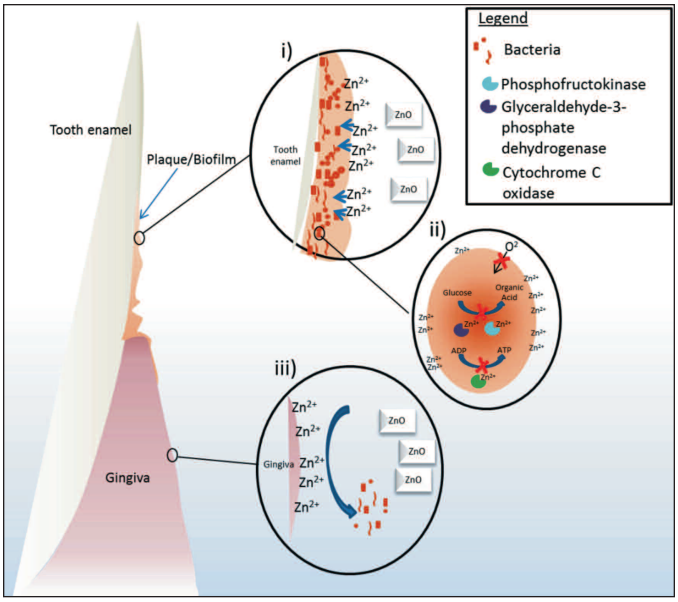

Figure 8. *The proposed antibacterial mode of action of zinc in the Dual Zinc plus Arginine dentifrice. (i) L-arginine in the dentifrice formula enhances the deposition, penetration, and retention of zinc in bacterial biofilms; (ii) the delivered zinc ions inhibit bacterial metabolic pathways limiting glycolysis, energy production, and respiration; (iii) zinc delivered to soft tissues can also limit bacterial adherence to these surfaces, enhancing resilience against microbial challenge.*

These collective findings suggest zinc may impact the oral microbiome by reducing the total bioload of periodontal pathogens. This impact on the overall virulence of the microbial community may be associated with a reduced risk of gum disease.

These studies have demonstrated that rational design of a dentifrice for enhanced delivery of an already effective active can translate to significant performance improvements. Building on zinc's well known effects in oral health, a new formula has been developed that improves the antibacterial profile of the active through uniquely modulating the reactivity and bioavailability of zinc. Leveraging the weaker binding constant of a more insoluble form of zinc (zinc oxide), a Dual Zinc dentifrice was developed. Greater biofilm control was observed in the Dual Zinc variants as a function of zinc oxide level as compared to treatment with a zinc citrate-only dentifrice. Through this Dual Zinc system, zinc oxide may serve as a prolonged source of zinc ions, potentially breaking down over time for continuous biofilm control. Enhancing oral bioavailability of the cation was targeted through developing a Dual Zinc plus Arginine dentifrice. L-arginine was chosen from a screening of amino acids based on a high positive charge and the potential to modulate active delivery via biofilm weakening or destabilization. Enhanced deposition, penetration, and retention of zinc in bacterial biofilm and on oral soft tissue *in vitro* was clear from multiple models. The enhanced zinc delivery upon treatment with the Dual Zinc plus Arginine dentifrice correlated with antimicrobial protection through reductions in bacterial metabolic function, reduced bacterial growth, and enhanced resistance of bacterial colonization on model oral soft tissues.

In later publications within this Special Issue, it will be demonstrated how effective delivery of zinc from the Dual Zinc plus Arginine dentifrice translates into effective long-term control of bacteria on teeth, tongue, cheeks, and gums, a 12-hour reduction in oral malodor, and significant reduction in plaque and gingivitis, all of which translate and contribute to improving whole mouth health.

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Conflict of Interest: All authors are employees of the Colgate-Palmolive Company.

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The Science of Developing Appealing Flavors to Drive Compliance

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Abstract

- **Objective:** To develop flavors for oral care formulations containing zinc oxide, zinc citrate and L-arginine that are stable for the toothpaste shelf life, mask the unpleasant astringency and metallic off notes of the base, have an appealing taste which pleases global consumers, stimulate regimen compliance, and therefore help deliver whole mouth health benefits to people throughout the world.
- **Methods:** For stability evaluation, flavor materials were formulated in Dual Zinc plus Arginine base and these samples were subjected to accelerated aging which consists of exposure to a temperature of 49°C for 6 weeks. The samples were analyzed by gas chromatography with flame ionization detector (GC FID) and gas chromatography mass spectrometry (GC MS) to confirm stability or establish changes in the chemical profile – loss of material and generation of degradation compounds. These samples were evaluated organoleptically by a flavor expert for taste acceptability and changes due to instability. Using state-of-the-art flavor expertise, tailor-made flavors were created. Their consumer appeal and acceptance were validated with monadic identified product tests. Their cooling attributes were evaluated by a panel of creative flavorists.
- **Results:** Certain classes of flavor molecules were not stable in the zinc and arginine-containing dentifrice. This significantly limited the choice of flavor materials that could be used to mitigate the undesirable taste of the dentifrice excipients and provide consumer acceptable taste. Through understanding of consumer expectations and needs, creative formulation using stable raw materials, and various novel cooling technologies, we were able to prepare flavors that successfully masked the unpleasant mouth sensation of the zinc and arginine-containing base. These specially designed flavors also provided impactful long-lasting cooling and freshness, thus complementing the toothpaste's therapeutic benefits. Consumer tests validated that these flavors had strong performance and acceptability among users of the original Colgate® Total® triclosan-containing dentifrice.
- **Conclusions:** Combining in-depth flavor scientific research and formulation creativity, we were able to deliver flavors that are stable and appealing to the global consumer for Colgate's new therapeutic segment.

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Introduction

Toothpaste is formulated to deliver oral care benefits ranging from prevention of caries, supragingival plaque, and calculus, in addition to breath freshness and relief of dentin hypersensitivity. The efficacy of the dentifrice is extremely important because it is tightly linked to increased consumer desire for improved oral health and well-being. To support whole mouth health, a toothpaste formulation might contain multiple excipients that work in synergy and complement each other. Along with efficacy, taste is another important attribute of oral care products because it is directly linked to consumer preference and usage. Formulations with pleasing and delightful taste drive consumer exposure to the novel products, strengthen the emotional connection with them, and ensure regimen compliance.[1,2] This translates into enhanced therapeutic value and efficacy for the consumers.

Taste is the sensory response to the fluid environment in the mouth.[3] It is mediated by groups of taste receptor cells clustered in layered balls known as taste buds. Hair-like extensions of the taste receptor cells protrude into a central pore at the top of the taste bud. The pore makes contact with the fluid environment in the mouth and the taste molecules interact with these extensions at or near the opening. These interactions involve surface proteins known as taste receptors or pore-like proteins called ion channels. Taste signals are transferred to the taste buds via protein binding (T1R receptor proteins for sweet and umami taste, T2R for bitter taste) or by changing cell membrane potential (for sour and salty taste). This causes chemical changes within the sensory cell that result in neural impulses being transmitted to the brain via different nerves. In the brain, the signal is decoded and taste is perceived.

Besides the five main classes of taste, a variety of chemically induced sensations can be perceived in the oral cavity.[4,5] These are described as chemesthetic sensations and occur through stimulation of the trigeminal nerves of the oral cavity. Many of the chemesthetic sensations are mediated by a special family of receptor proteins known as Transient Receptor Potential (TRP) channels.[6,7] Chemesthetic sensations that are specifically relevant to this flavor research are metallic and astringent. Metallic sensation arises from placing different metals or their salts in the mouth which triggers stimulation due to small changes in electrical potential.[8,9] Astringency is defined as "a complex sensation combining three distinct aspects: drying of the mouth, roughing of oral tissues, and pucker or drawing sensation felt in the cheeks and muscles of the face," and is associated with tannins as chemical stimuli.[10] The mechanism of astringency involves binding of the polyphenols to salivary proteins and mucins (slippery constituents of saliva), causing them to aggregate or precipitate, thus robbing the saliva of its ability to coat and lubricate oral tissues.[11] The result is dry, rough, and puckered sensations on oral tissues.

A novel toothpaste containing the breakthrough combination of Dual Zinc plus Arginine has been developed. Its efficacy has been

clinically proven to strongly support the whole mouth health benefits for consumers. However, the dentifrice base presents taste challenges due mainly to the presence of zinc-containing excipients as they impart undesirable off-notes to the toothpaste formulation. Zinc-containing molecules can interfere with the normal performance of the human taste system[12] by binding to the taste receptor proteins. In doing so, zinc can alter taste and inhibit chemesthetic stimuli of flavor ingredients added to the oral care formulation. Zinc can also bind to salivary proteins, which may alter in a negative way the ability of saliva to lubricate the oral mucosa, which imparts the sensation of astringency. Additionally, zinc oxide and zinc citrate impart a metallic sensation.

Good taste is delivered through good quality, high performing flavors. In a complex multicomponent system, another important factor to be considered is flavor stability. The presence of functional groups such as carbonyl, ester, and hydroxyl groups in the flavor molecules determines their possible chemical reactivity.[13] Even a flavor composition that is stable on its own can be susceptible to chemical changes due to interactions with the dentifrice molecules. For example, arginine amino and guanidino functional groups can possibly interact with flavor molecules, therefore causing flavor instability. It can decompose ester or organic carbonates through hydrolysis, or form condensation products with aldehydes. Such chemical changes impact overall flavor quality and affect taste consistency during the shelf life of the product.

The objective of our flavor research was to develop stable flavors for oral care formulations containing arginine, zinc oxide, and zinc citrate that overcome the unpleasant taste and mouth sensation imparted from ingredients in the base, and have a taste that global consumers like, which will drive appeal and daily compliance to ensure that therapeutic benefits are delivered as part of a daily oral hygiene regimen to help achieve whole mouth health.

Materials and Methods

An unflavored toothpaste formulation containing 0.96% zinc (zinc oxide, zinc citrate) and 1.5% L-arginine was used to study flavor materials stability. Flavor materials (individual components and essential oils) were provided by respective suppliers. These materials were added to the unflavored dentifrice base at typical use levels, ranging from 0.01 to 0.5%. Products used in consumer test studies were: original Colgate® Total® Advanced (UK) with triclosan toothpaste and Colgate® Dual Zinc plus Arginine therapeutic formulation with newly created flavors A, B, and C, all developed and manufactured by Colgate-Palmolive Company, New York, NY, USA.

Analytical Reagents

Isooctane (2, 2, 4-Trimethyl penthane) and Dichloromethane – gas chromatography grade were purchased from VWR. Sodium chloride – analytical grade was also obtained from VWR. Diphenyl ether used as internal standard was purchased from Sigma Aldrich.

Instrumentation

A Genie 2 vortex mixer was used to assist toothpaste sample dispersion. A Clinical 200 centrifuge (VWR) was used during the toothpaste sample preparations to enhance organic phase separation before chromatography analysis.

The Agilent 6890 gas chromatography system with flame ionization detector (GC FID) and on-column injector was used for the analysis of the toothpaste samples and respective standards of flavor ingredients. Separations were accomplished using a Supelcowax capillary column 100 m x 0.250 mm, d_f 0.25 µm or Supelcowax 30 m x 0.250 mm, d_f 0.25 µm. The injection volume was 0.5 ul and hydrogen was used as carrier gas. For Supelcowax 100 m column, the flow rate was 1.5 ml/min and the oven temperature program was 60°C, 3°C/min to 225°C, hold for 20 min. For Supelcowax 30 m column, we used programmed flow 1 ml/min (hold 5 min) to 5 ml/min at 0.1 ml/min/min, and the oven temperature program was 40°C to 230°C at 4°C/min, hold for 12.5 min. The detector conditions were: temperature 300°C, hydrogen flow 35 ml/min, and air flow 400 ml/min.

The GC MS system used was an Agilent chromatography system 6890 with 5973 mass selective detector equipped with split/splitless injector. Separations were accomplished using Supelcowax 30 m x 0.250, d_f 0.25 µm. Sample injection volume was 1 ul in split mode at ratio 1:10. The oven temperature program was 40°C to 230°C at 4°C/min. Helium was used as carrier gas at constant flow rate 1 ml/min. The MS source temperature was 230°C and the quat temperature was 150°C. The MS data were acquired in full scan mode (m/z 29-500).

Methods

The toothpaste samples containing the individual flavor materials were subjected to accelerated aging, which consists of exposure to a temperature of 49°C for six weeks, a testing protocol adopted from International Council for Harmonization (ICH) guidelines. Reference samples were stored at controlled room temperature 25°C, 60% relative humidity for six weeks. Profiling of the samples was done through organoleptic evaluations. Aged samples were organoleptically evaluated and compared to the reference samples by a trained flavor expert for taste acceptability or taste changes and off-notes due to instability.

Toothpaste samples containing the flavor materials before and after aging were prepared for chromatography analysis by suspending 1–3 g in saturated sodium chloride solution, followed by extraction with isooctane or dichloromethane solution, containing the internal standard. The extraction process was facilitated by thorough mixing using a vortexer. The organic and aqueous layers were separated by centrifugation at 3000 rpm for 5 min. Then, the organic layer was transferred into a 2 ml vial for analysis. The chromatography data (GC-FID and GC-MS) were used to evaluate degradation compounds and loss of flavor material through internal standard calculations.

Consumer test data were obtained from a study conducted in the United Kingdom. Products were tested in a two-week, monadic identified product test. Colgate Total Advanced triclosan-containing toothpaste (UK) was used as control (benchmark). The study was based on 150 respondents for each dentifrice product, selected to be known original Colgate Total triclosan users in the past 6 months. The tested flavor attributes were: overall flavor liking, length of flavor, aftertaste liking, and flavor strength during brushing. The action standards were overall opinion and the four statements: has a flavor I like, helps improve the health of my mouth, is a high quality product, leaves my mouth feeling clean and fresh. The toothpaste image attrib-

utes were: has a flavor that makes brushing more enjoyable, has a better flavor than other toothpaste I normally use, and freshness duration in my mouth. Top 1 box proportion for the control and each new flavor variant were calculated by dividing the number of respondents that selected the most favorable response by the total number of respondents. Top 2 box and Just about right proportions were calculated in the same way, but including, respectively, the responses to the two most favorable options and the neutral option. Then the percentage comparisons were calculated by dividing the Top 1 box, Top 2 box, and Just about right data obtained for each new formulation flavor option by the respective data obtained for the control. The cooling duration was evaluated by a five-member panel of creative flavorists.

Results

It was established that certain classes of flavor molecules were not stable in the novel dentifrice formulation. Table I summarizes initial and after aging experimental data for the levels of flavor materials that underwent significant degradation during aging. For example, methyl salicylate completely degraded at accelerated aging, resulting in the complete loss of wintergreen character on taste. The degradation of menthol propylene glycol carbonate (MPC) was approximately 50% at accelerated aging, which significantly diminished the cooling sensation. Aldehydes were another class of flavor compounds with low stability in the oral care formulations containing the amino acid. Our experimental results at accelerated aging showed the complete loss of ethyl vanillin and cinnamic aldehyde. Although these materials showed instability in the formulation, some essential oils and single flavor molecules were identified as being stable in the dentifrice base.

Table I
Concentrations of Flavor Compounds in Toothpaste Containing Dual Zinc plus Arginine Before and After Aging

Compound	Concentration Initial, ppm	Concentration After Aging, ppm
Methyl salicylate	4000	Not detected
Menthol propylene glycol carbonate (MPC)	950	440
Ethyl vanillin	45	3
Cinnamic aldehyde	500	5

Table II summarizes consumer test Top 1 box and Just about right results on the key flavor attributes: flavor liking, length of flavor, aftertaste liking, and flavor strength during brushing. The values for the proposed new flavor variants of the Dual Zinc plus Arginine dentifrice are expressed as percentage of the respective result for the Colgate Total Advanced triclosan-containing toothpaste. When the scores for flavor variants A and C were compared to the benchmark, they were higher or equal with no downsides. These were in the range 100–120% for dentifrice flavor option A and 102–110% for dentifrice flavor option C. Though still high, flavor B performed on overall flavor liking at 98% compared to the original Colgate Total triclosan-containing toothpaste. But it scored better or comparable on length of flavor, aftertaste liking, and flavor strength during brushing; 109%, 110%, and 100%, respectively.

Table II
Consumer Test Results on the Flavor Attributes for Flavor Variants of Dual Zinc plus Arginine Toothpaste Calculated as a Percentage of the Respective Results for the Control

Flavor attributes		Control[a]	New Formulation Flavor A	New Formulation Flavor B	New Formulation Flavor C
Consumer Sample Size	n	151	165	165	174
Overall flavor liking	Top 1	100%	116%	98%	104%
Length of flavor	Just right	100%	103%	109%	110%
Aftertaste liking	Top 1	100%	120%	110%	110%
Flavor strength during brushing	Just right	100%	100%	100%	102%

[a]The control is Colgate Total Advanced with triclosan (UK)

Table III presents a summary of consumer test results on the action standards and key statements for the proposed new flavor variants calculated as a percentage of the respective score for the Colgate Total Advanced triclosan-containing toothpaste. The newly created flavors delivered very strong Top 1 box action standard results, with options A and C outperforming the benchmark at 140% and 148%, respectively. Their Top 2 box scores on overall opinion were in the range 92%–101%. Though flavor B delivered at 92% on these criteria, it was the winning performer on all key statement metrics: has flavor I like, helps improve the health of the mouth, it's a high quality product, leaves my mouth feeling clean and fresh.

Table III
Consumer Test Results for the Action Standards for Flavor Variants of Dual Zinc plus Arginine Toothpaste Calculated as a Percentage of the Respective Results for the Control

Action standards		Control[a]	New Formulation Flavor A	New Formulation Flavor B	New Formulation Flavor C
Consumer Sample Size	n	151	165	165	174
Overall Opinion	Top 1	100%	140%	100%	148%
	Top 2	100%	95%	92%	101%
Key statements					
Has flavor I like	Top 2	100%	96%	101%	100%
Helps improve the health of the mouth	Top 2	100%	90%	100%	92%
It is high quality product	Top 2	100%	94%	101%	99%
Leaves my mouth feeling clean and fresh	Top 2	100%	99%	105%	105%

[a]The control is Colgate Total Advanced with triclosan (UK)

Table IV summarizes consumer test Top 1 box results on the image statements: has a flavor that makes brushing more enjoyable, has a

Table IV
Consumer Test Results on Image Attributes for Flavor Variants of Dual Zinc plus L-Arginine Toothpaste Calculated as a Percentage of the Respective Results for the Control

Image Statements		Control[a]	New Formulation Flavor A	New Formulation Flavor B	New Formulation Flavor C
Consumer Sample Size	n	151	165	165	174
Has a flavor that makes brushing more enjoyable	Top 1	100%	150%	96%	125%
Has a better flavor than other toothpaste I normally use	Top 1	100%	124%	100%	129%
Freshness duration in my mouth	Top 1	100%	114%	114%	121%

[a]The control is Colgate Total Advanced with triclosan (UK)

better flavor than other toothpaste I normally use, and freshness duration in my mouth. The data for the proposed new flavor variants of the Dual Zinc plus Arginine dentifrice were calculated as a percentage of the respective scores for the Colgate Total Advanced triclosan-containing paste. The new flavor options A and B achieved high scores respectively in the ranges 114–150% and 121–129%, which identified them as strong performers and demonstrated their significant contribution to the toothpaste consumer appeal.

Discussion

Creating flavors for the new therapeutic Dual Zinc plus Arginine dentifrice composition involved integration of many different flavor materials at various levels that could have numerous possible combinations. To study the stability of a full flavor in the base could be challenging and inconclusive. Our approach was to research the stability of individual flavor materials in the toothpaste formula in order to assess their potential use in the creative process. Through screening under accelerated aging conditions, the materials that underwent chemical and taste profile distortion and also the materials that did not change or impart off-notes were identified. It was confirmed that ester and organocarbonate flavor compounds were hydrolyzed and the reaction was greatly catalyzed by the presence of the amino acid. As discussed previously in flavor stability research,[14,15] for aldehyde containing flavor molecules, one probable reaction resulting in their instability could be Schiff base formation through condensation of the arginine amino group and the aldehyde group of the respective flavor molecule. Cinnamic aldehyde is a key material to deliver spicy notes, and ethyl vanillin is a key to the effective delivery of sweetness and creaminess. These tonalities can provide sensory changes in the mouth that mask astringency.[16] The instability of the compounds discussed above significantly limited the flavor creation palette that could be used to mitigate the undesirable taste of the dentifrice excipients and provide consumer-acceptable taste. Through understanding of consumer expectations, creative formulation using stable raw materials and various novel cooling technologies, we were able to prepare two generations of flavors that successfully masked the unpleasant mouth sensation of the zinc-containing base. The leading Generation I flavors were created with mint oils — peppermint, spearmint, and cornmint — along with other flavor ingredients, and a well-balanced addition of a novel cooling material or its combination with sensates, for example capsicum. The new cooling technology provided outstanding long lasting cooling up to 50 min without negative mouthfeel. This extended the astringent and metallic taste masking for a prolonged period of time after brushing. It delivered refreshing sensation, thus complementing the toothpaste's therapeutic benefits. These specially designed flavors met the expectations of the current users of Colgate Total triclosan-containing toothpaste. Analysis of the consumer data on the flavor attributes— flavor liking, length of flavor, aftertaste liking, and flavor strength during brushing— demonstrated consumer acceptability and appeal. The proposed new flavor options A and C met or exceeded the performance expectations of the benchmark standard. They were leading variants on the tested flavor attributes. The 98% score for flavor option B on overall flavor liking did not negatively affect its favorable position on length of flavor, aftertaste liking, and flavor strength during brushing.

In-depth and detailed analysis of the consumer data showed that these flavors had significantly contributed to the creation of a very favorable image profile for the new Colgate therapeutic toothpaste. They helped the brand to meet and exceed the action standard on "overall opinion" Top 1 box. The equal score for flavor option B, the significant increase of 40% for flavor option A and 48% for flavor option C when compared to the scores for the consumers using the triclosan-containing dentifrice, demonstrated the strength of the Dual Zinc plus Arginine therapeutic toothpaste. The acceptability of the new formulation with flavor option B was confirmed by the scores on key statements that were comparable to the benchmark toothpaste. Also, the high Top 1 box scores on image attributes indicated that consumers recognized that these flavors made brushing more enjoyable, they were better than those in the toothpaste they normally use, and that they provided freshness duration in the mouth.

To further enhance the performance of Generation I flavors, Generation II was created by incorporating a unique combination of two new cooling technologies. Thus, we were able to deliver a synergetic sensorial experience. This unique combination delivered enhanced cooling sensation; greater intensity and extended duration up to 60 min compared to Generation I flavors. Generation II flavors could be used successfully to support the advanced Dual Zinc plus Arginine therapeutic technology and further expand flavor offerings.

Our consumer test data clearly demonstrated that taste liking translates into consumer appeal and desire to use the product. As a result, therapeutic benefits would be delivered. Therefore, the pleasing taste would drive consumer compliance to the novel formulation and enhance whole mouth benefits.

Conclusions

The new Dual Zinc plus Arginine therapeutic technology presented significant challenges for flavor creation; *i.e.*, presence of strong base off-notes and instability of some flavor materials. The fundamental research that was conducted enabled us to identify the materials that were chemically unstable and changed, or lost their taste profile in the Dual Zinc plus Arginine formulation. Based on this in-depth knowledge, we built the palette for flavor creation comprising stable essential oils and individual ingredients, among them novel cooling technologies. Through good understanding of the taste profile of the therapeutic ingredients, we were able to artfully use this palette to design flavors for the Dual Zinc plus Arginine toothpaste that not only covered the unpleasant taste of the base, but also met the expectations of the current global users of triclosan-containing Colgate Total. These novel flavors were able to suppress the astringency and metallic taste of the therapeutic excipients and create a mouth sensation of freshness and clean feeling; drivers for consumer liking. Consumer test data showed that these tailor-made flavors supported a very favorable image of the new Colgate therapeutic segment and drove preference. These are essential prerequisites for compliance to regimen use and delivering the benefits of whole mouth health.

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The Effects of Two New Dual Zinc plus Arginine Dentifrices in Reducing Oral Bacteria in Multiple Locations in the Mouth: 12-Hour Whole Mouth Antibacterial Protection for Whole Mouth Health

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Abstract

- **Objective:** To compare the effects of two new fluoride toothpastes with Dual Zinc plus Arginine to the effects of a fluoride control toothpaste in reducing bacteria in oral biofilm on teeth and in multiple soft tissue locations, as well as in saliva, 12 hours after 14 and 29 days of product use.
- **Methods:** A randomized, single-center, three-cell, double-blind, parallel-group design was employed. The study protocol was approved by an Institutional Review Board. One hundred eighty adult subjects who met inclusion and exclusion criteria and signed an informed consent form were enrolled in the study. Subjects were randomly assigned to one of the three study products: 1) 0.96% zinc (zinc oxide, zinc citrate), 1.5% L-arginine and 1450 ppm fluoride as sodium fluoride in a silica base, Test 1; 2) 0.96% zinc (zinc oxide, zinc citrate), 1.5% L-arginine and 1000 ppm fluoride as sodium fluoride in a silica base, Test 2; and 3) 1450 ppm fluoride as sodium fluoride in a silica base, Control, for twice-daily use during tooth brushing. Oral samples were collected from the teeth, tongue, oral buccal mucosa, gingiva, and saliva at baseline and 12 hours after 14 and 29 days of assigned product use and were processed, serially diluted, plated, incubated, and scored for viable bacteria. Statistical analyses were performed separately for each sample site using ANOVA and ANCOVA for within- and between-treatment comparisons, respectively.
- **Results**: One hundred seventy-three subjects completed the study. Relative to subjects in the Control group, subjects in the two Test groups exhibited statistically significant reductions of 29–41% in numbers of bacteria in each of the five sample areas, 12 hours after 29 days of product use. Similar results were seen after 14 days of product use, but some differences were not statistically significant, indicating that the effects of these zinc-based toothpastes build over time with continued use. The two Test toothpastes were shown to be clinically equivalent using the Fieller's confidence interval test.
- **Conclusion:** Toothpastes containing 0.96% zinc (zinc oxide, zinc citrate), 1.5% L-arginine and either 1450 ppm or 1000 ppm fluoride as sodium fluoride in a silica base provide statistically significant reductions in oral bacteria on the teeth, tongue, cheeks, and gums, as well as in saliva, compared to toothpaste with fluoride alone, 12 hours after 29 days of twice-daily tooth brushing. The results demonstrate that regular and continued twice-daily use of these new toothpastes provide 12-hour whole mouth antibacterial protection for whole mouth health.

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Introduction

The role of dental plaque in the initiation and progression of oral disease, especially dental caries and periodontal disease, is well established, and the need to remove dental plaque on a regular and continued basis, through effective twice-daily tooth brushing and daily flossing, is also well known.[1] Unfortunately, many individuals are unable to achieve the high level of plaque removal required to completely prevent and control dental disease by mechanical oral hygiene procedures alone, so for most individuals the use of a clinically proven antibacterial fluoride toothpaste to supplement mechanical plaque removal is highly beneficial in enhancing the effectiveness of their tooth brushing routines and delivering better oral health outcomes.[2-5]

Research has demonstrated that the clinical efficacy of antibacterial oral care products in reducing dental plaque and gingivitis is driven by specific characteristics of the antibacterial agent and the product vehicle in which it is formulated. First, the antibacterial agent must possess innate biological properties that enable it to reduce the metabolism and growth of oral bacteria *in vivo*. Second, the antibacterial agent must be effectively released from the product matrix and delivered to the oral hard and soft tissues during use. Third, it must be retained on these surfaces and in dental plaque for sufficient time between oral hygiene occasions at a sufficient level to be able to exert its innate antibacterial properties.[6-8] Clinical studies on an essential oil-based mouthwash and on a fluoride toothpaste with triclosan/copolymer have confirmed the importance of these characteristics, and have demonstrated that statistically significant reductions in numbers of oral bacteria in dental plaque are concomitant with statistically significant reductions in dental plaque and gingivitis.[9,10] A more recent study has shown that reducing bacteria in both

hard and soft tissue sites throughout the whole mouth, not just on the teeth, but on the tongue, cheeks, and gingiva, is particularly beneficial to controlling dental plaque and improving gingival health.[11] The compelling logic to support this conclusion is that the oral biofilm present on the soft tissues is a reservoir of bacteria that can shed into saliva and transfer to recolonize the teeth. It follows that reducing oral biofilm on the soft tissues will result in improvements in plaque control and oral health, and there are data to support this logic.[12]

New thinking on the role of dental plaque in oral health and disease has led to the concept that the "normal" oral microbiome is beneficial to an individual's health and well-being, and should be maintained in its natural and balanced state as this promotes good oral health.[13,14] The corollary to this thinking is that in the future, new antibacterial oral care products should be developed to reduce oral biofilm by only "just enough" to reduce the risk of dental disease, while creating and supporting the beneficial functions of the "normal" oral microbiome consistent with health, and they should achieve both objectives through subtle effects on the oral biofilm that last for 12 hours between brushing occasions.[13,14] Based upon this new thinking, a new fluoride toothpaste containing a zinc-based antibacterial ingredient has been developed and validated.[12,15-19] Zinc was selected because it is clinically proven to reduce dental plaque and gingivitis and other plaque-related oral health benefits, and possesses the three key characteristics of clinically proven agents.[12] Zinc was also selected for its unexplored potential to strengthen and support the mouth's host defense through its vital role in maintaining physiological and metabolic processes in living tissues, such as defense against oxidative stress, support of regenerative processes, and maintenance of immune function.[12]

The new toothpaste contains Dual Zinc plus Arginine (0.96% zinc as zinc oxide and zinc citrate, and 1.5% L-arginine) and 1450 ppm fluoride (or 1000 ppm where local regulations require this level) as sodium fluoride in a silica base. The science underpinning this new toothpaste is discussed in the papers comprising this Special Issue publication, a brief summary of which follows to provide context to this study. The combination of Dual Zinc plus Arginine was selected on the basis of detailed studies of the chemistry and biological properties of this system for optimal delivery and retention of zinc and effective antibacterial activity *in vitro*.[15] The flavor of the toothpaste was optimized for stability, the effective masking of the astringency and metallic taste of zinc, consumer acceptability and liking, and signaling consistent with the Colgate therapeutic brand.[16] The clinical study reported in this paper confirms the selection of the Dual Zinc plus Arginine combination by demonstrating the delivery of 12-hour antibacterial protection throughout the whole mouth (whole mouth protection) *in vivo*. The results of this study are complemented by the results of a pivotal six-month clinical study which has proven that this new Dual Zinc plus Arginine fluoride toothpaste provides statistically significant reductions in established dental plaque and gingivitis,[17] the results of a three-week clinical study which have shown that the new toothpaste provides statistically significant reductions in oral malodor,[18] and the results of scientific and clinical studies confirming that breath freshening occurs through a dual mechanism of action.[19]

The aim of this clinical study was to evaluate the effects of brushing with a toothpaste containing 0.96% zinc (zinc oxide, zinc citrate), 1.5% L-arginine and 1450 ppm fluoride as sodium fluoride in a silica base (Test 1), and a toothpaste containing 0.96% zinc (zinc oxide, zinc citrate), 1.5% L-arginine and 1000 ppm fluoride as sodium fluoride in a silica base (Test 2), as compared to brushing with a toothpaste containing 1450 ppm fluoride as sodium fluoride in a silica base (Control) on numbers of oral organisms found in different oral sites, 12 hours after 14 and 29 days of product use.

Materials and Methods

This clinical study employed a randomized, single-center, three-cell, double-blind, parallel-group design. The study protocol was reviewed and approved by the SDM College of Dental Sciences and Hospital Institutional Review Board (IRB). One hundred eighty adult subjects from the Karnataka, India area, who met all the inclusion and exclusion criteria and signed an informed consent form, were enrolled into the clinical study.

Inclusion Criteria

Subjects had to be between the ages of 18–70. They were required to sign an informed consent form and demonstrate a willingness to comply with all study procedures and sampling schedules, including the ability to refrain from food for four hours and from drink for two hours prior to their clinical appointments. Subjects were required to possess a minimum of 20 natural teeth with facial and lingual scoreable surfaces, and have no overt signs of oral neglect. They also had to have a gingival index score greater than or equal to 1.0 (Löe-Silness Index) and a plaque index score greater than or equal to 1.5 (Turesky modification of Quigley-Hein Index), but not more than one periodontal pocket over 5 mm, and demonstrate adequate oral hygiene.

Exclusion Criteria

Subjects were excluded from the study if they had participated in any other clinical study or test panel, including clinical studies involving oral hygiene formulations, within the 30 days prior to entry into the study. They were also excluded if they had had a dental prophylaxis or treatment within the 30 days prior to the study, or had scheduled a dental prophylaxis or treatment or a medical procedure during the proposed study period. Subjects with significant oral soft tissue pathology, systemically related gingival enlargement, severe gingivitis (based on visual examinations), severe periodontal disease with bleeding gums and loose teeth, gross dental caries, severe generalized cervical abrasion and/or enamel abrasion, large fractured or temporary restorations (based on visual examinations), fixed or removable orthodontic appliance or removable partial dentures were also excluded. Subjects who had difficulty complying with study procedures and examinations, such as excessive gagging during oral assessments, a history of significant adverse effects following use of oral hygiene products, such as toothpaste and mouthwashes, allergies to personal care/consumer products or their ingredients, or to dental materials were also excluded. Subjects with a history of medical treatments, including antibiotic, anti-inflammatory, or anticoagulant therapy during the month preceding study enrollment, diabetes, hepatic or renal disease, or any other serious medical conditions or transmittable diseases (*e.g.*, heart disease or AIDS), rheumatic fever, heart murmur, mitral valve prolapse, or other conditions requiring prophylactic antibiotic coverage prior to invasive dental procedures, were excluded. Subjects who self-reported pregnancy or lactation, were known to be alcoholic or recovering alcoholic, had a history or were currently using recreational drugs, tobacco, or other habit promoting products were excluded, as were subjects with a history or current use of objects to pierce the lips or tongue.

Subjects were not permitted to use phenolic-flavored products, such as mint-flavored candies, chewing gums, wafers, mouthwashes, and other oral hygiene aids during the study period.

Clinical Procedures

Qualifying subjects and clinical study site personnel were blinded to product assignment. All products were over-wrapped with white tape in order to conceal product identity. Label information on each product consisted of a product code (study group code generated at Colgate and blinded to the clinical site personnel), instructions for at-home use and safety information, including emergency contact information. Study subjects were assigned a subject identification number in chronological order from 001 to 180 and were randomly assigned to one of the three treatment groups following a computer-generated randomization list.

Subjects were instructed to brush twice a day (morning and evening) for one minute with an approximately one-inch strip of their assigned toothpaste and the manual toothbrush provided (Colgate® Extra Clean, Colgate-Palmolive Company, New York, NY, USA) for a period of 29 days. Subjects were instructed to refrain from brushing on the morning of days 15 and 30, and from eating for four hours and drinking for two hours prior to their scheduled visit to the clinical facility to provide oral bacteria samples.

Clinical Scoring at Baseline

To determine whether subjects met the inclusion criteria for plaque and gingivitis, the following assessments were made prior to enrollment.

Dental Plaque Assessment. The dentition was disclosed and plaque scored at the disto-, mid-, mesio-buccal and disto-, mid-, mesio-lingual surfaces of each tooth according to the criteria of the modified Quigley and Hein Index (Turesky, *et al.* and Quigley and Hein) as follows.[20,21]

0 = No plaque;
1 = Separate flecks of plaque at the cervical margin;
2 = A thin, continuous band of plaque (up to 1 mm) at the cervical margin;
3 = A band of plaque wider than 1 mm, but covering less than 1/3 of the side of the crown of the tooth;
4 = Plaque covering at least 1/3, but less than 2/3 of the side of the crown of the tooth;
5 = Plaque covering 2/3 or more of the side of the crown of the tooth.

Subject-wise scores were calculated by summing all scores for all sites and dividing by the total number of sites scored.

Gingivitis Assessment. The degree of gingival inflammation was scored at six sites (disto-, mid-, mesio-buccal and disto-, mid-, mesio-lingual) of each tooth according to the criteria of the Gingival Index system outlined below (Löe and Silness).[22]

0 = Absence of inflammation;
1 = Mild inflammation – slight change in color and little change in texture;
2 = Moderate inflammation – moderate glazing, redness, edema, and hypertrophy;
3 = Severe inflammation – marked redness and hypertrophy. Tendency for spontaneous bleeding.

Subject-wise scores were calculated by summing all scores for all sites and dividing by the total number of sites scored.

Oral Bacteria Sampling

Supragingival Plaque. Samples were randomly collected from buccal surfaces of the upper right or left quadrant (teeth #s 2–8 or teeth #s 9–15) using a sterile Columbia 13/14 scaler, pooled and placed in a tube containing sterile isotonic phosphate buffered saline (PBS) and vortexed for 30 seconds to shake loose and disperse the collected oral sample.

Buccal and Tongue Scrapings. Separate samples were taken from each site using the edge of a disposable tongue blade (Puritan, Guilford, ME, USA). The investigator randomly collected from the right or left side of cheek and tongue. Each collection entailed five scrapes per site sampled. The tongue blades with oral samples were placed in a tube with 3 ml sterile suitable isotonic buffer, PBS, and vortexed for 30 seconds to shake loose and disperse the collected oral sample.

Gingival Scrapings. Samples were collected using a cytobrush, available commercially (Puritan, Guilford, ME). The investigator randomly collected from the entire arch of the gum. Each collection entailed five scrapes per site sampled. The brush with oral samples was placed in a tube with 3 ml sterile suitable isotonic buffer, PBS, and vortexed for 30 seconds to shake loose and disperse collected oral sample.

Oral Rinse Samples. Subjects were provided with 15 ml of saline in a sterile tube. Each subject rinsed with the provided buffer for 30 seconds and expectorated into a sterile tube marked with the subject information collected for analysis.

All samples were collected at each time-point (morning of days 15 and 30) and were transferred immediately to the microbiology laboratory where each sample was processed immediately upon receipt.

Microbiological Procedures

Oral bacteria samples were subjected to sonic oscillation using a Branson 450A sonicator with a Cup Horn for 30 seconds, pulsed (settings were Output=4, Duty cycle=50%), after which samples were serially diluted (10 fold) in PBS (Phosphate Buffered Saline). Dilutions from 10^0 to 10^5 were plated (on enriched agar with 5% sheep blood) according to manufacturer's directions on the media using a Spiral Systems Autoplate 4000 Spiral plate. Inoculated plates were incubated at 37°C and scored for viable bacteria. The colony forming units (CFU) were calculated from dilutions yielding at least 20 colonies per plate.

Oral Soft and Hard Tissue Assessment and Adverse Events

The dental examiner visually examined the oral cavity and perioral area using a dental light and dental mirror. This examination included an evaluation of the soft and hard palate, gingival mucosa, buccal mucosa, muco-gingival fold areas, tongue, sublingual and submandibular areas, salivary glands, and the tonsilar and pharyngeal areas. Adverse events were obtained from an interview with the each subject and from the dental examination of oral soft and hard tissues by the investigator.

Statistical Methods

The sample size calculation for this study utilized historical data from a previous study. It estimated that ~60 subjects should be included in each treatment group to detect a difference of

approximately 0.3 $\log_{10}$ CFU/ml in viable bacteria between treatments. Calculations were based on standard deviations recorded historically in similar studies with an α of 0.05 and statistical power of 80%. To normalize the data, the bacteria samples (CFU/ml) were logarithmically transformed (base$_{10}$) prior to statistical analysis with descriptive statistics presented as mean ± standard deviation at all evaluations.

Primary Analysis. Statistical analyses were performed separately on the oral buccal, supragingival plaque, gingiva, tongue, and salivary bacteria $\log_{10}$ CFU/ml data. Comparison of the treatment groups with respect to gender were performed using a Chi-Square analysis and for age, an analysis of variance (ANOVA). Comparisons of the treatment groups with respect to baseline levels of oral buccal, supragingival plaque, gingiva, tongue, and salivary bacteria $\log_{10}$ CFU/ml were performed using an analysis of variance (ANOVA). Within-treatment comparisons of the baseline levels versus follow-up levels of oral buccal, supragingival plaque, gingiva, tongue, and salivary bacteria $\log_{10}$ CFU/ml samples were performed using paired t-tests. Comparisons of the treatment groups with respect to baseline-adjusted levels of oral buccal, supragingival plaque, gingiva, tongue, and salivary bacteria $\log_{10}$ CFU/ml at the follow-up examinations were performed using analyses of covariance (ANCOVA) with baseline as the covariate. Post-ANCOVA pair-wise comparisons of the study treatments were performed using the Dunnett's test for multiple comparisons. All statistical tests of hypotheses were two-sided and employed a level of significance of $\alpha = 0.05$. Percent reductions were calculated throughout via the formula $(1\text{-}10^{diff}) \times 100$, where diff is equal to the difference between each treatment group or time points. A negative percent indicates an increase in bacteria.

Secondary Analysis. To demonstrate equivalency of the toothpaste containing 1450 ppm F (Test 1) and the toothpaste containing 1000 ppm F (Test 2) at the 15- and 30-day examinations, Fieller's Confidence Interval was used. Current guidelines indicate that the term "equivalent" is applicable if the mean oral bacteria values associated with Test 1 do not differ from those associated with Test 2 by more than 20% on the lower bound interval and 25% on the upper bound interval, *i.e.*, if the mean oral bacteria values associated with Test 1 are between 80% and 125% of those associated with Test 2.

Results

A CONSORT flow diagram showing the key steps in this study is provided in Figure 1.

Losses, Exclusions and Adverse Events

No adverse effects on the oral hard or soft tissues were observed by the examiner or reported by the study subjects when questioned at any time point during the study. Seven subjects did not complete the study period and were excluded from the study. The reasons for not completing the study were not product-related. Data analysis was carried out on the per protocol population of 173 compliant subjects.

Baseline Data

One hundred eighty subjects entered the clinical study and were randomized into one of the three treatment groups. One hundred seventy-three subjects complied with the protocol and completed the clinical study. A summary of the gender and age of the study population

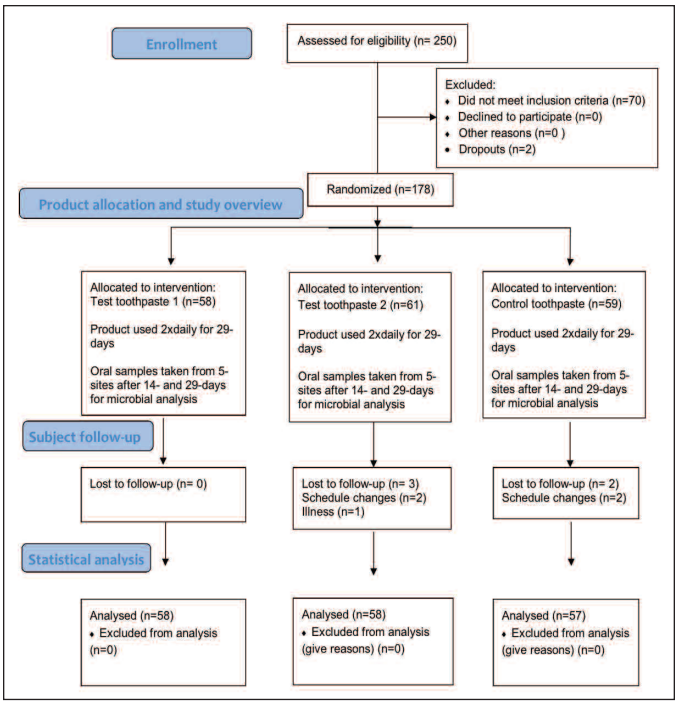

Figure 1. *CONSORT flow diagram showing the key steps in the study.*

is presented in Table I. The treatment groups did not differ significantly with respect to gender (p = 0.464), whereas the treatment groups did differ statistically significantly with respect to the age (p = 0.047).

Table II presents a summary of the mean buccal, supragingival plaque, gingiva, tongue, and salivary bacteria ($\log_{10}$ CFU/ml) data at the baseline, and at the examinations 12 hours after 14 days and 29 days of product use for those subjects who completed the clinical study. Statistically significant differences were observed among the treatment groups with respect to buccal, gingiva, and salivary bacteria ($\log_{10}$ CFU/ml) values at baseline (p= 0.014, p < 0.001, and p = 0.019, respectively), whereas no statistically significant differences were observed among the treatment groups with respect to supragingival plaque and tongue bacteria ($\log_{10}$ CFU/ml) values at baseline (p = 0.583 and p = 0.213, respectively).

Table I
Summary of Age and Gender Data for Subjects Who Completed the Clinical Study

	Number of Subjects			Age[5]	
Treatment	Male[4]	Female[4]	Total	Mean (SD)	Range
Test Toothpaste 1[1]	23	35	58	35.38 (10.73)	18–60
Test Toothpaste 2[2]	25	33	58	30.98 (10.34)	19–59
Control Toothpaste[3]	29	28	57	31.75 (9.25)	18–51

[1]A test toothpaste containing 0.96% zinc (zinc oxide and zinc citrate), 1.5% L-arginine and 1450 ppm fluoride as sodium fluoride in a silica base (Colgate-Palmolive Company, New York, USA)
[2]A test toothpaste containing 0.96% zinc (zinc oxide and zinc citrate), 1.5% L-arginine and 1000 ppm fluoride as sodium fluoride in a silica base (Colgate-Palmolive Company, New York, USA)
[3]A control toothpaste containing 1450 ppm fluoride as sodium fluoride in a silica base (Colgate-Palmolive Company, New York, USA)
[4]No statistically significant (p= 0.464) difference was indicated among the three treatment groups with respect to gender.
[5]A statistically significant (p= 0.047) difference was indicated among the three treatment groups with respect to age.

Results 12 Hours After 14 days of Product Use

Table III presents a summary of the baseline-adjusted mean buccal, supragingival plaque, gingiva, tongue, and salivary bacteria (log_{10} CFU/ml) data, together with the results of the within-treatment and between-treatment analyses 12 hours after 14 days of product use.

In summary, mean percent changes from baseline were always positive (reductions in bacteria) and statistically significant in each of the two Test groups and at each of the five oral sites sampled

Table II

Subject Mean (SD) Buccal, Supragingival Plaque, Gingiva, Tongue, and Salivary Bacteria (log_{10}CFU/ml) Values at Baseline, and 12 Hours after 14 Days and 29 Days Use for Subjects Who Completed the Clinical Study

Bacteria Source	Treatment	n	Baseline Mean (SD)	Mean (SD) After 14 Days' Use	Mean (SD) After 29 Days' Use
Buccal[4]	Test toothpaste 1[1]	58	6.67 (0.49)	6.51 (0.41)	6.52 (0.57)
	Test toothpaste 2[2]	58	6.67 (0.46)	6.52 (0.38)	6.55 (0.52)
	Control toothpaste[3]	57	6.46 (0.40)	6.51 (0.39)	6.52 (0.39)
Supragingival Plaque[5]	Test toothpaste 1[1]	58	6.77 (0.44)	6.68 (0.47)	6.62 (0.46)
	Test toothpaste 2[2]	58	6.80 (0.44)	6.70 (0.42)	6.69 (0.49)
	Control toothpaste[3]	57	6.71 (0.51)	6.74 (0.47)	6.76 (0.47)
Gingiva[6]	Test toothpaste 1[1]	58	6.87 (0.51)	6.79 (0.47)	6.72 (0.54)
	Test toothpaste 2 [2]	58	7.01 (0.55)	6.88 (0.53)	6.84 (0.62)
	Control toothpaste[3]	57	6.57 (0.46)	6.57 (0.45)	6.60 (0.44)
Tongue[7]	Test toothpaste 1[1]	58	6.80 (0.46)	6.65 (0.43)	6.65 (0.53)
	Test toothpaste 2[2]	58	6.78 (0.49)	6.65 (0.43)	6.64 (0.50)
	Control toothpaste[3]	57	6.65 (0.45)	6.61 (0.48)	6.74 (0.52)
Saliva[8]	Test toothpaste 1[1]	58	7.27 (0.49)	7.15 (0.50)	7.04 (0.48)
	Test toothpaste 2[2]	58	7.44 (0.47)	7.32 (0.51)	7.21 (0.52)
	Control toothpaste[3]	57	7.17 (0.55)	7.17 (0.49)	7.19 (0.49)

[1]A test toothpaste containing 0.96% zinc (zinc oxide and zinc citrate), 1.5% L-arginine and 1450 ppm fluoride as sodium fluoride in a silica base (Colgate-Palmolive Company, New York, USA).
[2]A test toothpaste containing 0.96% zinc (zinc oxide and zinc citrate), 1.5% L-arginine and 1000 ppm fluoride as sodium fluoride in a silica base (Colgate-Palmolive Company, New York, USA).
[3]A control toothpaste containing 1450 ppm fluoride as sodium fluoride in a silica base (Colgate-Palmolive Company, New York, USA).
[4]A statistically significant (p= 0.014) difference was indicated among the three treatment groups at baseline with respect to buccal bacteria (log_{10}CFU/ml) levels.
[5]No statistically significant (p= 0.583) difference was indicated among the three treatment groups at baseline with respect to supra-gingival plaque bacteria (log_{10}CFU/ml) levels.
[6]A statistically significant (p< 0.001) difference was indicated among the three treatment groups at baseline with respect to gingiva bacteria (log_{10}CFU/ml) levels.
[7]No statistically significant (p= 0.213) difference was indicated among the three treatment groups at baseline with respect to tongue bacteria (log_{10}CFU/ml) levels.
[8]A statistically significant (p = 0.019) difference was indicated among the three treatment groups at baseline with respect to salivary bacteria (log_{10}CFU/ml) samples.

Table III

Baseline-Adjusted Subject Mean (SE) Buccal, Supragingival Plaque, Gingiva, Tongue, and Salivary Bacteria (log_{10}CFU/ml) Values 12 Hours after 14 Days of Product Use for Subjects Who Completed the Clinical Study

Bacteria Source	Treatment	n	Adj. Mean (SE) Use After 14 Days	Within-Treatment Analysis		Between-Treatment vs. Control Toothpaste	
				% Change[4]	Sig.[5]	% Difference[6]	Sig[7]
Buccal	Test toothpaste 1[1]	58	6.46 (0.03)	30.8%	p < 0.001	30.8%	p < 0.001
	Test toothpaste 2[2]	58	6.46 (0.03)	29.2%	p < 0.001	30.8%	p < 0.001
	Control toothpaste[3]	57	6.62 (0.03)	-12.2%	p = 0.041	----	----
Supragingival Plaque	Test toothpaste 1[1]	58	6.67 (0.02)	18.7%	p = 0.001	24.1%	p = 0.002
	Test toothpaste 2[2]	58	6.67 (0.02)	20.6%	p = 0.001	24.1%	p = 0.001
	Control toothpaste[3]	57	6.79 (0.02)	-7.2%	p = 0.135	----	----
Gingiva	Test toothpaste 1[1]	58	6.74 (0.03)	16.8%	p = 0.021	8.8%	p = 0.652
	Test toothpaste 2[2]	58	6.71 (0.03)	25.9%	p = 0.001	14.9%	p = 0.261
	Control toothpaste[3]	57	6.78 (0.03)	0.0%	p = 0.778	----	----
Tongue	Test toothpaste 1[1]	58	6.61 (0.03)	29.2%	p < 0.001	14.9%	p = 0.198
	Test toothpaste 2[2]	58	6.62 (0.03)	25.9%	p = 0.004	12.9%	p = 0.327
	Control toothpaste[3]	57	6.68 (0.03)	8.8%	p = 0.238	----	----
Saliva	Test toothpaste 1[1]	58	7.17 (0.03)	24.1%	p < 0.001	20.6%	p = 0.094
	Test toothpaste 2[2]	58	7.20 (0.04)	24.1%	p < 0.001	14.9%	p = 0.226
	Control toothpaste[3]	57	7.27 (0.04)	0.0%	p = 0.918	----	----

[1]A test toothpaste containing 0.96% zinc (zinc oxide and zinc citrate), 1.5% L-arginine and 1450 ppm fluoride as sodium fluoride in a silica base (Colgate-Palmolive Company, New York, USA).
[2]A test toothpaste containing 0.96% zinc (zinc oxide and zinc citrate), 1.5% L-arginine and 1000 ppm fluoride as sodium fluoride in a silica base (Colgate-Palmolive Company, New York, USA).
[3]A control toothpaste containing 1450 ppm fluoride as sodium fluoride in a silica base (Colgate-Palmolive Company, New York, USA).
[4]% change exhibited by the mean after 14-days use relative to the baseline mean. A positive value indicates a reduction in bacteria (log_{10}CFU/ml) after 14-days use.
[5]Significance of paired t-test comparing the baseline and the examination after 14-days use.
[6]Difference between the means expressed as a % of mean for the control toothpaste after 14-days use. A positive value indicates a reduction in bacteria (log_{10}CFU/ml) relative to control toothpaste.
[7]Significance of the post-ANCOVA Dunnett's test comparison of baseline-adjusted means.

[buccal: 30.8% (p < 0.001) – Test 1, 29.2% (p < 0.001) – Test 2; supragingival plaque: 18.7% (p=0.001) – Test 1, 20.6% (p = 0.001) – Test 2; gingiva: 16.8% (p = 0.021) – Test 1, 25.9% (p = 0.001) – Test 2; tongue: 29.2% (p < 0.001) – Test 1, 25.9% (p = 0.004) – Test 2; salivary bacteria: 24.1% (p < 0.001) – Test 1, 24.1% (p < 0.001) – Test 2]. In contrast, mean percent changes from baseline in the Control group were variable (two increases, two no change), although they were generally not statistically significant [buccal: -12.2% (p = 0.041); supragingival plaque: -7.2% (p = 0.135); gingiva: 0.0% (p = 0.778); tongue: 8.8% (p = 0.238); salivary bacteria: 0.0% (p = 0.918)]. In addition, relative to subjects in the Control group, subjects in each of the two Test groups exhibited numeric reductions in bacteria at each of the five oral sites sampled, but not all were statistically significant [buccal: 30.8% (p < 0.001) – Test 1, 30.8% (p < 0.001) – Test 2; supragingival plaque: 24.1% (p = 0.002) – Test 1, 24.1% (p = 0.001) – Test 2; gingiva: 8.8% (p = 0.652) – Test 1, 14.9% (p = 0.261) – Test 2; tongue: 14.9% (p = 0.198) – Test 1, 12.9% (p = 0.327) – Test 2; salivary bacteria: 20.6% (p = 0.094) – Test 1, 14.9% (p = 0.226) – Test 2].

Results 12 Hours After 29 Days of Product Use

Table IV presents a summary of the baseline-adjusted mean buccal, supragingival plaque, gingiva, tongue, and salivary bacteria (log_{10} CFU/ml) data, together with the results of the within-treatment and between-treatment analyses 12 hours after 29 days of product use.

Once again, mean percent changes from baseline were always positive (reductions in bacteria) and statistically significant in each of the two test groups and at each of the five oral sites sampled [buccal: 29.2% (p < 0.001) – Test 1, 24.1% (p < 0.001) – Test 2; supragingival plaque: 29.2% (p < 0.001) – Test 1, 22.4% (p = 0.015) – Test 2; gingiva: 29.2% (p = 0.004) – Test 1, 32.4% (p < 0.001) – Test 2; tongue: 29.2% (p < 0.001) – Test 1, 27.6% (p = 0.001) – Test 2; salivary bacteria: 41.1% (p < 0.001) – Test 1, 41.1% (p < 0.001) – Test 2]. Mean percent changes from baseline in the Control group were consistently negative (increases in bacteria) and were generally not statistically significant [buccal: -14.8% (p = 0.078); supragingival plaque: -12.2% (p = 0.0.045); gingiva: -7.2% (p = 0.180); tongue: -23.0% (p = 0.0.065); salivary bacteria: -4.7% (p = 0.614)]. Furthermore, relative to subjects in the Control group, subjects in each of the two Test groups exhibited statistically significant reductions in bacteria at each of the five oral sites sampled [buccal: 35.4% (p<0.001) - Test 1, 32.4% (p=0.001) - Test 2; supragingival plaque: 38.3% (p<0.001) - Test 1, 29.2% (p=0.004) - Test 2; gingiva: 25.9% (p=0.043) - Test 1, 27.6% (p=0.038) - Test 2; tongue: 39.7% (p=0.001) - Test 1, 38.3% (p<0.001) - Test 2; salivary bacteria: 41.1% (p<0.001) - Test 1, 38.3% (p=0.001) - Test 2].

Figure 2 illustrates the results of the study. For simplicity, it shows the baseline-adjusted mean levels of buccal, supragingival plaque, gingiva, tongue, and salivary bacteria (log_{10} CFU/ml) data collected 12 hours after 29 days of product use for the negative control and one of the new Dual Zinc plus Arginine toothpastes with 0.96% zinc (zinc oxide, zinc citrate), 1.5% L-arginine and 1450 ppm fluoride as sodium fluoride in a silica base (Test 1).

Test of Product Equivalence

The results of the two Fieller confidence interval tests indicate that the two Test toothpastes are clinically equivalent 12 hours after 14 and 29 days of twice-daily use.

Table IV

Baseline-Adjusted Subject Mean (SE) Buccal, Supragingival Plaque, Gingiva, Tongue, and Salivary Bacteria (log_{10}CFU/ml) Values 12 Hours After 29 Days of Product Use for Subjects Who Completed the Clinical Study

Bacteria Source	Treatment	n	Adj. Mean (SE) Use After 14 Days	Within-Treatment Analysis		Between-Treatment vs. Control Toothpaste	
				% Change[4]	Sig.[5]	% Difference[6]	Sig.[7]
Buccal	Test toothpaste 1[1]	58	6.46 (0.03)	29.2%	p < 0.001	35.4%	p < 0.001
	Test toothpaste 2[2]	58	6.48 (0.03)	24.1%	p < 0.001	32.4%	p = 0.001
	Control toothpaste[3]	57	6.65 (0.04)	-14.8%	p = 0.078	----	----
Supragingival Plaque	Test toothpaste 1[1]	58	6.60 (0.03)	29.2%	p < 0.001	38.3%	p < 0.001
	Test toothpaste 2[2]	58	6.66 (0.03)	22.4%	p = 0.015	29.2%	p = 0.004
	Control toothpaste[3]	57	6.81 (0.03)	-12.2%	p = 0.045	----	----
Gingiva	Test toothpaste 1[1]	58	6.68 (0.04)	29.2%	p = 0.004	25.9%	p = 0.043
	Test toothpaste 2[2]	58	6.67 (0.04)	32.4%	p < 0.001	27.6%	p = 0.038
	Control toothpaste[3]	57	6.81 (0.04)	-7.2%	p = 0.180	----	----
Tongue	Test toothpaste 1[1]	58	6.60 (0.04)	29.2%	p < 0.001	39.7%	p = 0.001
	Test toothpaste 2[2]	58	6.61 (0.04)	27.6%	p = 0.001	38.3%	p = 0.001
	Control toothpaste[3]	57	6.82 (0.04)	-23.0%	p = 0.065	----	----
Saliva	Test toothpaste 1[1]	58	7.06 (0.04)	41.1%	p < 0.001	41.1%	p < 0.001
	Test toothpaste 2[2]	58	7.09 (0.04)	41.1%	p < 0.001	38.3%	p = 0.001
	Control toothpaste[3]	57	7.29 (0.04)	-4.7%	p = 0.614	----	----

[1]A test toothpaste containing 0.96% zinc (zinc oxide and zinc citrate), 1.5% L-arginine and 1450 ppm fluoride as sodium fluoride in a silica base (Colgate-Palmolive Company, New York, USA.).

A test toothpaste containing 0.96% zinc (zinc oxide and zinc citrate), 1.5% L-arginine and 1000 ppm fluoride as sodium fluoride in a silica base (Colgate-Palmolive Company, New York, USA).

[3]A control toothpaste containing 1450 ppm fluoride as sodium fluoride in a silica base (Colgate-Palmolive Company, New York, USA).

[4]% change exhibited by the mean after 29-days us relative to the baseline mean. A positive value indicates a reduction in bacteria (log_{10}CFU/ml) at the 29-day examination.

[5]Significance of paired t-test comparing the baseline and the examinations after 29-days use

[6]Difference between the means expressed as a % of the mean for the control toothpaste after 29-days use. A positive value indicates a reduction in bacteria (log_{10}CFU/ml) relative to control toothpaste.

[7]Significance of the post-ANCOVA Dunnett's test comparison of baseline-adjusted means.

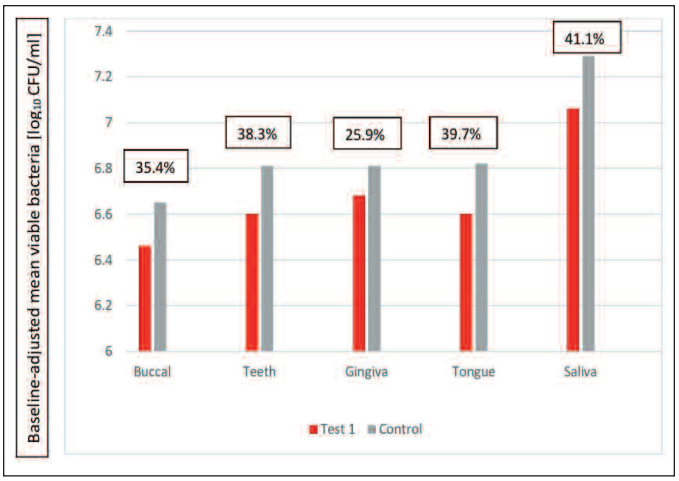

Figure 2. *The effects on numbers of oral bacteria in five oral microenvironments, the teeth, tongue, cheeks (buccal) and gums (gingiva), as well as saliva, of brushing with a new Dual Zinc plus Arginine toothpaste containing 0.96% zinc (zinc oxide, zinc citrate), 1.5% L-arginine and 1450 ppm fluoride (Test 1) compared to brushing with a toothpaste containing 1450 ppm fluoride alone (Control), 12-hours after 29-days of twice daily tooth brushing. Oral bacteria levels are shown on the y axis as baseline-adjusted means (log_{10}CFU/ml). The comparison of results for the Test 1 versus Control groups for each of the five oral sites is shown on the x axis. % reductions for each site are shown as the difference between the baseline-adjusted means of the Test 1 and Control groups expressed as a % of the baseline-adjusted mean for the Control group.*

Summary and Conclusions

This randomized, double-blind, three-cell, clinical study has demonstrated that a toothpaste containing Dual Zinc plus Arginine (0.96% zinc as zinc oxide and zinc citrate, 1.5% L-arginine) and 1450 ppm fluoride as sodium fluoride in a silica base (Test 1) and a toothpaste containing Dual Zinc plus Arginine (0.96% zinc as zinc oxide and zinc citrate, and 1.5% L-arginine) and 1000 ppm fluoride as sodium fluoride in a silica base (Test 2) provide statistically significant reductions in oral bacteria on multiple hard and soft tissue sites throughout the mouth, 12 hours after 29 days product use, compared to a control toothpaste containing 1450 ppm fluoride as sodium fluoride in a silica base alone. At this time point, subjects in the Test 1 and Test 2 groups exhibited statistically significant ($p < 0.001$ and $p = 0.001$) mean reductions in oral buccal bacteria of 35.4% and 32.4%, respectively; statistically significant ($p < 0.001$ and $p = 0.004$) mean reductions in oral supragingival plaque bacteria of 38.3% and 29.2%, respectively; statistically significant ($p = 0.043$ and $p = 0.038$) mean reductions in oral gingiva bacteria of 25.9% and 27.6%, respectively; statistically significant ($p = 0.001$) mean reductions in oral tongue bacteria of 39.7% and 38.3%, respectively; and statistically significant ($p < 0.001$ and $p = 0.001$) mean reductions in oral salivary bacteria of 41.1% and 38.3%, respectively, relative to subjects in the Control group. In addition, the two Test toothpastes were shown to be clinically equivalent using the Fieller confidence interval test 12 hours after 14 and 29 days of use.

The results of this clinical study are highly consistent with those previously reported for a zinc citrate toothpaste which showed statistically significant reductions in oral bacteria on hard and soft tissue surfaces, and in saliva, in the range of 23–38%, overnight after an evening brushing and five hours after a morning brushing.[23] Fundamental to achieving an effective ecological approach to dental plaque control and the prevention and control of gingivitis is the concept of reducing oral bacteria in biofilms sufficiently to reduce disease risk, while supporting the beneficial functions of biofilms consistent with health. Relative to the well-established antibacterial fluoride toothpaste with triclosan/copolymer,[11] this new Dual Zinc plus Arginine fluoride toothpaste provides moderate reductions in oral bacteria yet, importantly, it provides statistically significant reductions in established plaque and gingivitis of ~30% and ~26%, respectively, relative to a regular fluoride toothpaste control which are quantitatively comparable with the statistically significant reductions in established plaque and gingivitis of 22% and 22%, respectively, reported in a systematic review and meta-analysis of the triclosan/copolymer toothpaste studies.[4]

The scientific and clinical studies presented in this Special Issue support that this new Dual Zinc plus Arginine fluoride toothpaste conforms to recently articulated concepts of the "normal" oral microbiome and its importance as a natural and balanced state in oral health. This new toothpaste reduces oral bacteria on the hard and soft tissues sufficiently to significantly reduce established dental plaque and gingivitis, while creating and supporting beneficial functions of the "normal" oral microbiome consistent with health, such as preventing colonization of exogenous, often pathogenic, microbes and down-regulating potentially damaging pro-inflammatory responses, achieving both through subtle effects on the oral microbiome that last for 12 hours between brushing occasions.

A holistic approach to effective prevention and good oral health is both timely and appropriate. This innovative Dual Zinc plus Arginine fluoride toothpaste offers patients and consumers whole mouth protection against future oral challenges and whole mouth health. As part of a risk-based preventive program which embraces the core elements of patient-centered care, this toothpaste can empower patients to achieve effective prevention and good oral health. In addition, with direct-to-consumer messaging that motivates and empowers consumers to improve self-care, this toothpaste can also enhance prevention and improve oral health for everyone who uses it.

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Conflict of Interest: Drs. KVV Prasad, SG Therathil, and A Agnihotri are independent clinical investigators in the Department of Public Health Dentistry, SDM College of Dental Sciences and Hospital, Karnataka, India. Mr. LR Mateo is an independent statistical consultant at LRM Statistical Consulting LLC, West Orange, New Jersey, USA. Dr. Prasad and Mr. Mateo declare no conflict of interest. Dr. PK Sreenivasan is an employee of the Colgate-Palmolive Company. Dr. D Cummins is a retiree of the Colgate-Palmolive Company. She is currently an independent consultant and was funded by the Colgate-Palmolive Company to author this paper.

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A Clinical Investigation of a Dual Zinc plus Arginine Dentifrice in Reducing Established Dental Plaque and Gingivitis Over a Six-Month Period of Product Use

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Abstract

- **Objective**: The objective of this single-center, double-blind, parallel-group, randomized six-month clinical study was to evaluate the clinical efficacy of a new Dual Zinc plus Arginine dentifrice (Colgate-Palmolive Co., New York, NY, USA) containing zinc (zinc oxide, zinc citrate) 0.96%, 1.5% Arginine, and 1450 ppm fluoride as sodium fluoride in a silica base in reducing established dental plaque and gingivitis over a six-month period, relative to that of a regular fluoride dentifrice containing 1450 ppm fluoride as sodium fluoride in a silica base (Colgate-Palmolive Co., New York, NY, USA).
- **Methods**: A total of 100 adult male and female subjects from Santo Domingo, Dominican Republic were enrolled in this clinical study. During the baseline visit, the dental examiner clinically measured three gingival parameters (gingival index, gingival severity index, gingival interproximal index) and three plaque parameters (plaque index, plaque severity index, plaque interproximal index). The examining clinician also performed an assessment of the oral soft and hard tissues. All subjects were then assigned a subject identification number in chronological order from 001 to 100 and were randomly assigned to one of two treatment groups following a computer-generated randomization list. They were provided with their assigned dentifrice and an adult, soft-bristled toothbrush for home use, and were instructed to brush twice daily (morning and evening) for one minute with the assigned dentifrice for a period of six months. Subjects returned to the study facility site for their follow-up evaluation of plaque and gingivitis parameters after three and six months.
- **Results:** Ninety-six (96) subjects completed the study. At the three-month evaluation, subjects in the Dual Zinc plus Arginine dentifrice group exhibited statistically significant ($p < 0.001$) reductions in all gingival and plaque parameters relative to subjects in the fluoride dentifrice group. For gingival parameters, reductions were 18.8% for gingival index, 33.3% for gingival severity index, and 19.1% for gingival interproximal index. For plaque parameters, reductions were 11.0% for plaque index, 22.4% for plaque severity index, and 9.8% for plaque interproximal index. At the six-month evaluation, subjects in the Dual Zinc plus Arginine dentifrice group presented continuous statistically significant ($p < 0.001$) reductions in all three gingival and plaque parameters when compared to the subjects in the fluoride dentifrice group. For gingival parameters, reductions were 26.3% for gingival index, 56.6% for gingival severity index, and 29.2% for gingival interproximal index. For plaque parameters, reductions were 30.1% for plaque index, 61.9% for plaque severity index, and 28.0% for plaque interproximal index after six months of product use,
- **Conclusions**: The overall results of this double-blind clinical study support the conclusion that a Dual Zinc plus Arginine dentifrice containing zinc (zinc oxide, zinc citrate) 0.96%, 1.5% Arginine, and 1450 ppm fluoride as sodium fluoride in a silica base provides significantly greater reduction in dental plaque and gingivitis parameters as compared to a regular fluoride dentifrice containing 1450 ppm fluoride as sodium fluoride in a silica base after three months and six months of product use.

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Introduction

Looking broadly, academic journals dedicated to researching health, lifestyles, public policy, and actions aim to bring knowledge to the medical community as well as trickling it down to the average person.[1-4] From nutrition to supplements to lifestyles, consumers are able to discern a causal link to health.

However, one area that is seemingly being overlooked by consumers focused on their own health is that of oral health. While poor oral health is one of the key factors that can affect an individual's psychosocial well-being and capacity in biting, chewing, smiling, and speaking,[5] it requires special attention. Through concerted efforts of dental professionals, patients are becoming more educated. They begin to understand that oral bacteria and dental plaque should be effectively removed in order to keep the mouth healthy. If not removed regularly, plaque will eventually lead to major issues such as malodor, caries, and gum disease,[6,7] and will increase an individual's risk of possible systemic implications.[8-12] In addition, bacteria in saliva adhered

to soft tissue can colonize/re-colonize[13] the teeth and lead to the continued cycle of oral health problems.

While multiple methods and products exist to aid consumers in managing oral health, tooth brushing is a daily ritual that remains the most common method of oral care. It is estimated that approximately 80–90% of the population in developed countries uses a toothbrush once or twice a day.[14,15] However, even this habitual and effective daily routine does not guarantee prevention of oral issues as often plaque is not effectively removed from all the surfaces of the oral cavity where it resides.

The impact of which surfaces have the highest propensity for bacteria to grow in the oral cavity can be understood better by looking at the following surface area numbers. The mean surface area of the adult oral cavity is approximately 215 cm^2.[16] The teeth account for 20% of the surface area, while keratinized and non-keratinized soft tissues comprise about 30% and 50% of this surface area, respectively.[17] The soft tissues comprising about 80% of the mouth can harbor a significant reservoir of bacteria from which the biofilm on all oral surfaces is repopulated. To support this, a number of studies have shown that soft tissue surfaces harbor a significant number of periodontal pathogens.[18-29] These data indicate that the soft tissues of the oral cavity are an important reservoir for periodontal pathogens and could be a major factor in the re-colonization of tooth surfaces after therapeutic procedures.[17]

Management of bacterial colonization and re-colonization should include the combination of mechanical and chemical approaches. There are several ways to achieve antiplaque activity of toothpastes, such as preventing bacterial adhesion, limiting bacterial growth/colonization, disrupting an already established biofilm, or by altering the composition and/or pathogenicity of the biofilm.[30] Inclusion of effective antibacterial agents in dentifrices is a viable approach to managing the bacteria that the mechanical act of tooth brushing has missed.

As previously outlined by Manus,[31] *et al*., a combination of zinc citrate, zinc oxide, and Arginine have demonstrated utility in various *in vitro* experiments, including zinc delivery and biofilm reduction. Zinc is ubiquitous in the human body[32] and has been used in dentifrice formulations for plaque control, oral malodor reduction, and for calculus formation reduction through the inhibition of crystal growth.[33-35] Arginine has been shown to bind to negatively charged surfaces.[36] Further, 1.5% Arginine has already been demonstrated through *in vitro* experiments to enhance zinc solubility and increase the deposition of zinc to model surfaces.

The purpose of this study was to evaluate the clinical efficacy of a new dentifrice containing Dual Zinc plus Arginine formula in a silica base as compared to a regular fluoride dentifrice in reducing dental plaque and gingivitis over a six-month period of product use.

Materials and Methods

Subjects and Study Design

This clinical study was performed in Santo Domingo, Dominican Republic. It employed a randomized, single-center, double-blind, and parallel-group design. A total of 100 adult male and female subjects were enrolled into the study based upon the following inclusion and exclusion criteria. Inclusion criteria: (i) Subjects had to be between the ages of 18 and 70 (inclusive), in general good health, and possess at least 20 uncrowned permanent natural teeth (excluding third molars); (ii) Subjects were required to be available for the six-month duration of the study, and to sign an informed consent form; (iii) Subjects were required to present with an initial mean Gingival Index score of at least 1.0 as determined by the Löe-Silness Gingival Index, and an initial mean Plaque Index score of at least 1.5 as determined by the Turesky modification of the Quigley-Hein Plaque Index.

Exclusion criteria: (i) Subjects were excluded from participation in the study if they had orthodontic bands, partial removable dentures, tumor(s) of the soft or hard tissues of the oral cavity, advanced periodontal disease (purulent exudates, tooth mobility and/or extensive loss of periodontal attachment or alveolar bone), or five or more decayed carious lesions requiring immediate restorative treatment; (ii) Subjects were excluded from the study if they had a history of allergies to oral care / personal care consumer products or their ingredients, if they presented an existing medical condition that prohibits the cessation of food and drink consumption for four hours, if they used antibiotics anytime during the one month prior to entry into the study, or were using any other prescription medicines that might interfere with the study outcome. Pregnant or lactating women were excluded from participation, as well as subjects with a history of alcohol or drug abuse; (iii) Subjects were also excluded from the study if they participated in any other clinical study or test panel within the one month prior to entry into the study, or if they had received a dental prophylaxis in the past two weeks prior to the baseline examination.

The sample size of 100 subjects (50 per group) was determined based on the standard deviation for the response measures of 0.30, a significance level of $\alpha = 0.05$, a 10% attrition rate, and an 80% power level, which allowed this study to detect a minimal statistically significant difference between the study groups means of 15%. The sample size calculation utilized historical data from a previous study.

Qualifying subjects who met the inclusion/exclusion criteria were assigned a subject identification number in chronological order from 001 to 100 and were randomly assigned to one of two treatment groups following a computer-generated randomization list.

Following study treatment assignment, subjects were provided with their assigned dentifrice and an adult, soft-bristled toothbrush for home use. They were instructed to brush twice daily (morning and evening) for one minute with approximately 1.5 grams of the dentifrice for a period of six months.

Qualifying subjects, as well as all clinical study site personnel, were blinded to product assignment; products were covered with white over-wrapping paper in order to conceal product identity. Label information on each tube consisted of a toothpaste code (study group code), instructions for at-home use, and safety information, including emergency contact information.

Dentifrices Tested

For the purpose of this report, the treatments are identified as follows:

Test Dentifrice: a Dual Zinc plus Arginine dentifrice containing zinc (zinc oxide, zinc citrate) 0.96%, 1.5% Arginine, and 1450 ppm fluoride as sodium fluoride in a silica base (Colgate-Palmolive Co., New York, NY, USA).

Control Dentifrice: a regular fluoride dentifrice containing 1450 ppm fluoride as sodium fluoride in a silica base (Colgate-Palmolive Co., New York, NY, USA).

Clinical Scoring Procedures

Gingivitis Assessment. To evaluate the degree of gingival inflammation, each tooth was divided into six surfaces and the Gingival Index (GI) was scored in accordance with the Löe-Silness Gingival Index criteria.[37] Subject-wise whole mouth scores were calculated summing all scores for all sites and dividing by the total number of sites scored.

Gingival Severity. To evaluate Gingival Severity, GI scores of 2 and 3 at baseline were included in the calculation. Subject-wise, the whole mouth scores were calculated by counting the scores for the sites with baseline GI = 2 and 3 and dividing by the total number of sites assessed.

Gingival Interproximal. To evaluate Gingival Interproximal, GI scores at (1) mesio-buccal, (3) disto-buccal, (4) mesio-lingual, and (6) disto-lingual were included in the calculation. Subject-wise, the whole mouth scores were calculated summing the scores for sites 1, 2, 4, and 6 and dividing by the total number of sites.

Dental Plaque Assessment. To evaluate Plaque Index (PI), the dentition was disclosed with disclosing solution and plaque scored on each of the tooth surfaces according to the Turesky modification of the Quigley-Hein Plaque Index.[38] Subject-wise, whole mouth scores were calculated summing all scores for all sites and dividing by the total number of sites scored.

Plaque Severity. To evaluate Plaque Severity, baseline PI scores of (3) disto-buccal, (4) mesio-lingual, and (5) mid-lingual surfaces were included in the calculation. Subject-wise, whole mouth scores were calculated counting the PI scores for sites 3-5 and dividing by the total number of sites assessed.

Plaque Interproximal. To evaluate Plaque Interproximal, baseline PI scores at (1) mesio-buccal, (3) disto-buccal, (4) mesio-lingual, and (6) disto-lingual surfaces were included in the calculation. Subject-wise, the whole mouth scores were calculated summing the PI scores for sites 1, 2, 4, and 6 and dividing by the total number of sites.

Oral Soft and Hard Tissue Assessment. The dental examiner visually examined the oral cavity and peri-oral area using a dental light and dental mirror. This examination included an evaluation of the soft and hard palate, gingival mucosa, buccal mucosa, mucogingival fold areas, tongue, sublingual and submandibular areas, salivary glands, tonsilar and pharyngeal areas, and the teeth.

Adverse Events. Adverse events were obtained from an interview with the subject and from an oral examination by dental examiner.

Statistical Methods

Statistical analyses were performed separately for the gingivitis assessments and dental plaque assessments. Comparisons of the treatment groups with respect to gender were performed using a Chi-Square analysis, and for age using an Independent t-test. Comparisons of the treatment groups with respect to baseline gingival index scores and plaque index scores were performed using an Independent t-test. Within-treatment comparisons of the baseline versus follow-up gingival and plaque index scores were performed using paired t-tests. Comparisons of the treatment groups with respect to baseline-adjusted gingival and plaque scores at the follow-up examinations were performed using analyses of covariance (ANCOVA). All statistical tests of hypotheses were two-sided, and employed a level of significance of $\alpha = 0.05$.

Ethics

The study protocol was reviewed and approved by the Concordia Research Institutional Review Board (IRB).

Results

Ninety-six subjects out of one hundred successfully completed the study in accordance with the protocol. Four subjects did not complete the study for reasons unrelated to the use of the study treatments and were excluded from the data analyses. The treatment groups did not differ statistically significantly with respect to the gender ($p = 0.317$) and age ($p = 0.164$) characteristics. A summary of the gender and age of the study population is presented in Table I.

Table I
Summary of Age and Gender for Subjects
Who Completed the Clinical Study

Treatment	Number of Subjects			Age	
	Male	Female	Total	Mean ± SD	Range
Test Dentifrice[1]	17	32	49	32.06 ± 10.70	19-57
Control Dentifrice[2]	21	26	47	35.23 ± 11.47	19-63
All Treatment Groups	38	58	96	33.61 ± 11.14	19-63

1. A Dual Zinc plus Arginine dentifrice containing zinc (zinc oxide, zinc citrate) 0.96%,1.5% Arginine, and 1450 ppm F as NaF in a silica base (Colgate-Palmolive Co., New York, NY, USA).
2. A regular fluoride dentifrice containing 1450 ppm fluoride as NaF in a silica base (Colgate-Palmolive Co., New York, NY, USA).

Baseline Data

Table II presents a summary of the Gingival Index, Gingival Severity Index, and Gingival Interproximal Index scores measured at the baseline evaluations for those subjects who completed the six-month clinical study. For Gingival Index, the mean baseline scores were 2.15 for the Test Dentifrice group and 2.06 for the Control Dentifrice group. For Gingival Severity Index, the mean baseline scores were 0.80 for the Test Dentifrice group and 0.76 for the Control Dentifrice group. For Gingival Interproximal Index, the mean baseline scores were 2.28 for the Test Dentifrice group and 2.18 for the Control Dentifrice group.

No statistically significant difference was detected among the two treatment groups at baseline examination with respect to either

Table II
Summary of the Baseline Gingival Index, Gingival Severity Index, and Gingival Interproximal Index Scores
For Subjects Who Completed the Six-Month Clinical Study

Index	Treatment	n	Baseline (Mean ± SD)
Gingival	Test Dentifrice[1]	49	2.15 ± 0.44
	Control Dentifrice[2]	47	2.06 ± 0.41
Gingival Severity	Test Dentifrice	49	0.80 ± 0.18
	Control Dentifrice	47	0.76 ± 0.17
Gingival Interproximal	Test Dentifrice	49	2.28 ± 0.44
	Control Dentifrice	47	2.18 ± 0.41

1. A Dual Zinc plus Arginine dentifrice containing zinc (zinc oxide, zinc citrate) 0.96%, 1.5% Arginine, and 1450 ppm F as NaF in a silica base (Colgate-Palmolive Co., New York, NY, USA).
2. A regular fluoride dentifrice containing 1450 ppm fluoride as NaF in a silica base (Colgate-Palmolive Co., New York, NY, USA).

Gingival Index (p = 0.312), Gingival Severity Index (p = 0.314), or Interproximal Gingival Index (p=0.257) scores.

The Plaque Index, Plaque Severity Index, and Plaque Interproximal Index scores measured at the baseline evaluations are found in Table III. For Plaque Index, the mean baseline scores were 2.81 for the Test Dentifrice group and 2.82 for the Control Dentifrice group. For the Plaque Severity Index, the mean baseline scores were 0.65 for the Test Dentifrice group and 0.65 for the Control Dentifrice group. For the Plaque Interproximal Index, the mean baseline scores were 3.05 for the Test Dentifrice group and 3.10 for the Control Dentifrice group.

No statistically significant difference was indicated among the two treatment groups at baseline examination with respect to either Plaque Index (p = 0.885), Plaque Severity Index (p = 0.761), or Plaque Interproximal Index (p = 0.557) scores.

Table III

Summary of the Baseline Plaque Index, Plaque Severity Index, and Plaque Interproximal Index Scores For Subjects Who Completed the Six-Month Clinical Study

Index	Treatment	n	Baseline (Mean ± SD)
Plaque	Test Dentifrice[1]	49	2.81 ± 0.40
	Control Dentifrice[2]	47	2.82 ± 0.36
Plaque Severity	Test Dentifrice	49	0.65 ± 0.16
	Control Dentifrice	47	0.65 ± 0.13
Plaque Interproximal	Test Dentifrice	49	3.05 ± 0.38
	Control Dentifrice	47	3.10 ± 0.37

1. A Dual Zinc plus Arginine dentifrice containing zinc (zinc oxide, zinc citrate) 0.96%, 1.5% Arginine, and 1450 ppm F as NaF in a silica base (Colgate-Palmolive Co., New York, NY, USA).
2. A regular fluoride dentifrice containing 1450 ppm fluoride as NaF in a silica base (Colgate-Palmolive Co., New York, NY, USA).

Three-Month Data – Gingival Parameters

Table IV summarizes the baseline-adjusted mean Gingival Index, Gingival Severity Index, and Gingival Interproximal Index scores measured after three months of twice-a-day brushing with the assigned product for those subjects who completed the six-month clinical study.

Relative to the baseline data, the Test Dentifrice group and the Control Dentifrice group showed statistically significant (p < 0.001) reductions of 30.7% and 12.6%, respectively, in Gingival Index scores. Comparing the two treatment groups, the Test Dentifrice group exhibited an 18.8% improvement in the Gingival Index scores relative to Control Dentifrice at a 95% confidence level. Statistical reductions for the Gingival Severity Index scores for both the Test Dentifrice group and the Control Dentifrice group (p < 0.001 and p = 0.001, respectively) of 43.8% and 14.5%, respectively, were measured after three months. Consistent with results from the Gingival Index scores, subjects from the Test Dentifrice group exhibited a statistically significant improvement of 33.3% in the Gingival Severity index score (p < 0.001) in comparison to the Control Dentifrice after three months of product use.

Further, statistically significant (p < 0.001) reductions of 30.7% and 11.9% in Gingival Interproximal Index scores for both the Test Dentifrice group and the Control Dentifrice group were measured. However, a reduction of 19.1% (p < 0.001) comparing the Test Dentifrice group to the Control Dentifrice group was measured.

Three-Month Data – Plaque Parameters

Table V presents a summary of the baseline-adjusted mean Plaque Index, Plaque Severity Index, and Plaque Interproximal Index scores measured after three months of twice-a-day brushing with the assigned product for those subjects who completed the six-month clinical study. It should be noted that a negative change from baseline indicates an increase in the index score.

The Plaque Index, Plaque Severity Index, and Plaque Interproximal Index were all measured against the baseline. The Test Dentifrice demonstrated a significant reduction on all three indexes with measurements of 11.0% (p < 0.001) on Plaque, 20% (p < 0.001) on Plaque Severity, and 10.2% (p < 0.001) on Plaque Interproximal versus baseline. Contrary to this, the Control Dentifrice did not have any statistically significant reductions on any of the indexes with measurements of 0.4% (p = 0.866) on Plaque, -1.5% (p = 0.411) on Plaque Severity, and 1.3% (p = 0.385) on Plaque Interproximal, respectively.

When comparing the Test Dentifrice to the Control Dentifrice group, a significant reduction across all three indexes was already apparent at three months. The relative reduction compared to the Control Dentifrice was 11.0% (p < 0.001) on Plaque, 22.4% (p < 0.001) on Plaque Severity, and 9.8% (p < 0.001) on Plaque Interproximal measurements, respectively.

Table IV

Baseline-Adjusted Subject Mean (SE) Gingival Index, Gingival Severity Index, and Gingival Interproximal Index Scores at the Three-Month Examination For Subjects Who Completed the Six-Month Clinical Study

Index	Treatment	N	Adj. Three-Month Mean (SE)	Within-Treatment Analysis		Between-Treatment Comparison	
				Percent Change[3]	Sig.[4]	Percent Difference[5]	Sig.[6]
Gingival	Test Dentifrice[1]	49	1.47 ± 0.04	30.7%	p < 0.001	18.8%	p < 0.001
	Control Dentifrice[2]	47	1.81 ± 0.04	12.6%	p < 0.001		
Gingival Severity	Test Dentifrice	49	0.44 ± 0.03	43.8%	p < 0.001	33.3%	p < 0.001
	Control Dentifrice	47	0.66 ± 0.03	14.5%	p = 0.001		
Gingival Interproximal	Test Dentifrice	49	1.57 ± 0.04	30.7%	p < 0.001	19.1%	p < 0.001
	Control Dentifrice	47	1.94 ± 0.04	11.9%	p < 0.001		

1. A Dual Zinc plus Arginine dentifrice containing zinc (zinc oxide, zinc citrate) 0.96%, 1.5% Arginine and 1450 ppm F as NaF in a silica base (Colgate-Palmolive Co., New York, NY).
2. A regular fluoride dentifrice containing 1450 ppm fluoride as NaF in a silica base (Colgate-Palmolive Co., New York, NY).
3. Percent change exhibited by the three-month mean relative to the baseline mean. A positive value indicates a reduction in index scores at the three-month examination.
4. Significance of paired t-test comparing the baseline and the three-month examinations.
5. Difference between the three-month means expressed as a percentage of the three-month mean for the Control Dentifrice. A positive value indicates a reduction in index scores for the Test Dentifrice relative to Control Dentifrice.
6. Significance of ANCOVA comparison of baseline-adjusted three-month means.

Six-Month Data – Gingival Parameters

Table VI presents a summary of the baseline-adjusted mean Gingival Index, Gingival Severity Index, and Gingival Interproximal Index scores measured after six months of twice-a-day brushing with the assigned product for those subjects who completed the six-month clinical study.

The Gingival Index, Gingival Severity Index, and Gingival Interproximal Index were all measured against the baseline. The Test Dentifrice demonstrated a significant reduction on all three indexes with measurements of 46.5.0% ($p < 0.001$) on Gingival, 71.3% ($p < 0.001$) on Gingival Severity and 46.5% ($p < 0.001$) on Gingival Interproximal versus baseline. Contrary to this, the Control Dentifrice had statistically significant reductions on each of the indexes with much lower measurements of 24.3% ($p < 0.001$) on Gingival, 30.3% ($p < 0.001$) on Gingival Severity and 22.0% ($p < 0.001$) on Gingival Interproximal, respectively.

When comparing the Test Dentifrice to the Control Dentifrice group, significant reduction across all three indexes had increased from the three-month measurements. The relative reduction compared to the Control Dentifrice was 26.3% ($p < 0.001$) on Gingival, 56.6% ($p < 0.001$) on Gingival Severity, and 29.2% ($p < 0.001$) on Gingival Interproximal measurements, respectively.

Six-Month Data – Plaque Parameters

Table VII presents a summary of the baseline-adjusted mean Plaque Index, Plaque Severity Index, and Plaque Interproximal Index scores measured after six months of twice-a-day brushing with the assigned product for those subjects who completed the six-month clinical study.

The Plaque Index, Plaque Severity Index, and Plaque Interproximal Index were all measured against the baseline. The Test Dentifrice demonstrated a significant reduction on all three indexes with measurements of 32.4% ($p < 0.001$) on Plaque, 63.1% ($p < 0.001$) on Plaque Severity, and 31.1% ($p < 0.001$) on Plaque Interproximal versus baseline. Contrary to this, the Control Dentifrice did not have statistically significant reductions on the first two indexes with measurements of 3.5% ($p = 0.051$) on Plaque, 3.1% ($p = 0.448$) on Plaque Severity; however, there was a significant reduction of 5.2% ($p = 0.004$) on Plaque Interproximal, respectively.

When comparing the Test Dentifrice to the Control Dentifrice group, a significant reduction across all three indexes was already

Table V

Baseline-Adjusted Subject Mean (SE) Plaque Index, Plaque Severity Index and Plaque Interproximal Index Scores at the Three-Month Examination For Subjects Who Completed the Six-Month Clinical Study

Index	Treatment	N	Adj. Three-Month Mean (SE)	Within-Treatment Analysis Percent Change[3]	Within-Treatment Analysis Sig.[4]	Between-Treatment Comparison Percent Difference[5]	Between-Treatment Comparison Sig.[6]
Plaque	Test Dentifrice[1]	49	2.50 ± 0.04	11.0%	p < 0.001	11.0%	p < 0.001
	Control Dentifrice[2]	47	2.81 ± 0.04	0.4%	p < 0.866		
Plaque Severity	Test Dentifrice	49	0.52 ± 0.02	20.0%	p < 0.001	22.4%	p < 0.001
	Control Dentifrice	47	0.67 ± 0.02	-1.5%	p = 0.411		
Plaque Interproximal	Test Dentifrice	49	2.75 ± 0.04	10.2%	p < 0.001	9.8%	p < 0.001
	Control Dentifrice	47	3.05 ± 0.04	1.3%	p < 0.385		

1. A Dual Zinc plus Arginine dentifrice containing zinc (zinc oxide, zinc citrate) 0.96%, 1.5% Arginine, and 1450 ppm F as NaF in a silica base (Colgate-Palmolive Co., New York, NY, USA).
2. A regular fluoride dentifrice containing 1450 ppm fluoride as NaF in a silica base (Colgate-Palmolive Co., New York, NY, USA).
3. Percent change exhibited by the three-month mean relative to the baseline mean. A positive value indicates a reduction in index scores at the three-month examination.
4. Significance of paired t-test comparing the baseline and the three-month examinations.
5. Difference between the three-month means expressed as a percentage of the three-month mean for the Control Dentifrice. A positive value indicates a reduction in index scores for the Test Dentifrice relative to Control Dentifrice.
6. Significance of ANCOVA comparison of baseline-adjusted three-month means.

Table VI

Baseline-Adjusted Subject Mean (SE) Gingival Index, Gingival Severity Index and Gingival Interproximal Index Scores at the Six-Month Examination For Subjects Who Completed the Six-Month Clinical Study

Index	Treatment	N	Adj. Three-Month Mean (SE)	Within-Treatment Analysis Percent Change[3]	Within-Treatment Analysis Sig.[4]	Between-Treatment Comparison Percent Difference[5]	Between-Treatment Comparison Sig.[6]
Gingival	Test Dentifrice[1]	49	1.15 ± 0.04	46.5%	p < 0.001	26.3%	p < 0.001
	Control Dentifrice[2]	47	1.56 ± 0.04	24.3%	p < 0.001		
Gingival Severity	Test Dentifrice	49	0.23 ± 0.03	71.3%	p < 0.001	56.6%	p < 0.001
	Control Dentifrice	47	0.53 ± 0.03	30.3%	p = 0.001		
Gingival Interproximal	Test Dentifrice	49	1.21 ± 0.04	46.5%	p < 0.001	29.2%	p < 0.001
	Control Dentifrice	47	1.71 ± 0.04	22.0%	p < 0.001		

1. A Dual Zinc plus Arginine dentifrice containing zinc (zinc oxide, zinc citrate) 0.96%, 1.5% Arginine, and 1450 ppm F as NaF in a silica base (Colgate-Palmolive Co., New York, NY, USA).
2. A regular fluoride dentifrice containing 1450 ppm fluoride as NaF in a silica base (Colgate-Palmolive Co., New York, NY, USA).
3. Percent change exhibited by the six-month mean relative to the baseline mean. A positive value indicates a reduction in index scores at the six-month examination.
4. Significance of paired t-test comparing the baseline and the 6-month examinations.
5. Difference between the six-month means expressed as a percentage of the six-month mean for the Control Dentifrice. A positive value indicates a reduction in index scores for the Test Dentifrice relative to Control Dentifrice.
6. Significance of ANCOVA comparison of baseline-adjusted six-month means.

Table VII
Baseline-Adjusted Subject Mean (SE) Plaque Index, Plaque Severity Index, and Plaque Interproximal Index Scores at the Six-Month Examination For Subjects Who Completed the Six-Month Clinical Study

Index	Treatment	N	Adj. Three-Month Mean (SE)	Within-Treatment Analysis		Between-Treatment Comparison	
				Percent Change[3]	Sig.[4]	Percent Difference[5]	Sig.[6]
Plaque	Test Dentifrice[1]	49	1.90 ± 0.05	32.4%	p < 0.001	30.1%	p < 0.001
	Control Dentifrice[2]	47	2.72 ± 0.05	3.5%	p < 0.051		
Plaque Severity	Test Dentifrice	49	0.24 ± 0.02	63.1%	p < 0.001	61.9%	p < 0.001
	Control Dentifrice	47	0.63 ± 0.02	3.1%	p = 0.448		
Plaque Interproximal	Test Dentifrice	49	2.11 ± 0.05	31.1%	p < 0.001	28.0%	p < 0.001
	Control Dentifrice	47	2.93 ± 0.05	5.2%	p < 0.004		

1. A Dual Zinc plus Arginine dentifrice containing zinc (zinc oxide, zinc citrate) 0.96%, 1.5% Arginine, and 1450 ppm F as NaF in a silica base (Colgate-Palmolive Co., New York, NY, USA).
2. A regular fluoride dentifrice containing 1450 ppm fluoride as NaF in a silica base (Colgate-Palmolive Co., New York, NY, USA).
3. Percent change exhibited by the six-month mean relative to the baseline mean. A positive value indicates a reduction in index scores at the six-month examination.
4. Significance of paired t-test comparing the baseline and the six-month examinations.
5. Difference between the six-month means expressed as a percentage of the six-month mean for the Control Dentifrice. A positive value indicates a reduction in index scores for the Test Dentifrice relative to Control Dentifrice.
6. Significance of ANCOVA comparison of baseline-adjusted six-month means.

apparent at six months. The relative reduction compared to the Control Dentifrice was 30.1% ($p < 0.001$) on Plaque, 61.9% ($p < 0.001$) on Plaque Severity, and 28.0% ($p < 0.001$) on Plaque Interproximal measurements, respectively.

Discussion and Conclusions

When the health of the oral cavity is driven more by the need for balance than overall sterility, the importance of whole mouth health becomes clear. It is important to limit the growth of bacteria to prevent overgrowth and eventual dysbiosis, but some level of beneficial and/or non-harmful species are needed to maintain the generalized health of the soft tissues and prevent overgrowth and dysbiosis.[39] To this end, it is the overall maintenance of both the balance of plaque and the health of the soft tissue and related immune response that defines a truly healthy mouth. In this context, large-scale killing of oral bacteria is not necessarily desirable and, while effective at eliminating disorders in the short term, does not promote long-term health of the entire oral system.

Maintaining healthy gums is a vital component of establishing whole mouth health. In the clinical trial reported in this study, the effect of a new toothpaste containing the combination of zinc (zinc oxide, zinc citrate) 0.96%, and 1.5% arginine on gingival health and plaque control was assessed. The developed dentifrice has been shown to effectively treat biofilms *in vitro*.[31] A clinical trial has also shown the new toothpaste containing dual zinc with arginine was significantly effective at reducing the levels of bacteria found on the oral hard and soft tissue surface that represent the whole mouth's surfaces.[40,41] This study demonstrates that the effects of this new toothpaste on bacteria that were observed both *in vitro* and clinically translated into a clinically relevant end benefit of improved gingival health and plaque control.

As demonstrated by Manus,[31] *et al*. in laboratory studies, the combination of zinc salts with arginine is an effective delivery system of zinc, and the use of arginine enhances delivery and retention relative to a zinc dentifrice without arginine. However, the question remained if such a combination of zinc salts and arginine would offer plaque and gingivitis reductions over prolonged use *in vivo*.

The clinical study presented in this paper examined the effect on plaque and gingivitis by a dentifrice containing a Dual Zinc plus Arginine system. The effectiveness of the dentifrice was examined at both three- and six-month time points. As pointed out previously, zinc is used throughout the body in various biological processes, including many enzymatic reactions. The biological relevance of zinc makes the possibility of sequestering zinc a potential concern. Sequestering would, obviously, lead to an impairment of the intended oral benefits. The hypothesis was that if the Dual Zinc plus Arginine system was bioavailable to reduce bacteria, there would be a statistical effect on both the plaque and gingivitis levels of the test subjects.

One of the key attributes of an effective therapeutic toothpaste is the ability to show improvements in multiple measures related to gingival health, such as overall plaque and gingival indexes, as well specific measures related to severity and hard-to-reach places (interproximal). To that end, the new Dual Zinc plus Arginine toothpaste demonstrates its efficacy in as little as three months, with statistical reductions in plaque and gingival index scores at three months, which continue to build through six months leading to significant reductions in Plaque Index scores (30.1%) and Gingival Index scores (26.3%) compared to subjects from the regular fluoride dentifrice group. Reductions in plaque severity (61.9%) and interproximal plaque indexes (28.0%) also improved, which translated into improvements in reduced gingival severity index scores (56.6%), and in gingival interproximal index scores (29.2%) compared to the regular fluoride dentifrice group.

Additionally, the ability of the Dual Zinc plus Arginine toothpaste to reverse gum problems at sites with more severe gingivitis (bleeding) was demonstrated in this study. The Gingival Severity Index is a subset analysis of the overall Löe-Silness Gingival Index that is used to assess the extent of gingival bleeding reduction by measuring the most severe gingival sites starting with a baseline Löe-Silness Gingival Index score of 2 or 3 (with bleeding) that improve over the clinical study to a score of 0 or 1 (without gingival bleeding). Compared to baseline, usage of the Dual Zinc plus Arginine toothpaste resulted in a 71.3% reduction of gingival severity (bleeding) after six months usage while the fluoride-only toothpaste resulted in a 30.3% reduction. Thus, usage of the Dual Zinc plus Arginine toothpaste resulted in not only an overall reduction in gingivitis, but also reversal

and recovery of the severe gingival bleeding sites to no bleeding after six months. Additionally, compared to baseline, usage of the Dual Zinc plus Arginine toothpaste resulted in a 46.5% reduction of gingivitis from an average baseline gingival index score of 2.15 to 1.15 after six months usage. This change reflects a reversal from moderate to more mild inflammation on the Löe-Silness Gingival Index scoring scale. In contrast, usage of the fluoride only toothpaste resulted in only a 24.3% reduction of gingival index from a baseline gingival index of 2.06 to 1.56 after six months.

This study confirms that the newly formulated Dual Zinc plus Arginine system offers a clinical benefit in plaque and gingivitis reductions, two key aspects of overall oral health. While zinc is known to function by a completely different mechanism than triclosan, it is important to note that not only is efficacy demonstrated, but the new Dual Zinc plus Arginine system offers plaque and gingivitis benefits on par with those from triclosan.[42] The overall results of this double-blind clinical study further support the conclusion that a Dual Zinc plus Arginine dentifrice containing zinc (zinc oxide, zinc citrate) 0.96%, 1.5% arginine, and 1450 ppm fluoride as sodium fluoride in a silica base provided a significantly greater reduction in dental plaque and gingivitis parameters as compared to a regular fluoride dentifrice containing 1450 ppm fluoride as sodium fluoride in a silica base after three months and six months of product use.

In conclusion, we have clinically proven plaque and gingivitis benefits of this new dentifrice utilizing Dual Zinc plus Arginine. This new dentifrice provides an additional tool for consumers to address their overall health starting in the mouth by effectively controlling plaque bacteria, which are the underlying cause of gum-related problems and other oral conditions and diseases.

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Conflict of Interest: ED, MR, and Y-PZ are employed by the Colgate-Palmolive Company. The other authors declare that they have no conflicts of interest.

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A Clinical Investigation of the Efficacy of a Dual Zinc plus Arginine Dentifrice for Controlling Oral Malodor

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Abstract

- **Objective**: The objective of this independent, double-blind clinical study was to assess the efficacy of a new Dual Zinc plus Arginine dentifrice (Colgate-Palmolive Co., New York, NY, USA) containing zinc (zinc oxide, zinc citrate) 0.96%, 1.5% arginine, and 1450 ppm F as sodium fluoride in a silica base for the 12-hour overnight oral malodor reduction after three weeks of product use, relative to that of a regular fluoride dentifrice containing 1450 ppm F as sodium fluoride in a silica base (Colgate-Palmolive Co., New York, NY, USA).
- **Methods**: A total of eighty (80) adult male and female subjects from Chengdu, People's Republic of China, were enrolled in this clinical study. Following an assessment of the oral soft and hard tissues, subjects were evaluated for baseline oral malodor by a panel of four trained and calibrated judges using a nine-point organoleptic hedonic scale. They were then randomly assigned to one of two treatment groups (Dual Zinc plus Arginine – test; regular fluoride dentifrice – control). Subjects were provided with their assigned dentifrice and toothbrush and instructed to brush their teeth twice daily (morning and evening) for one minute. After three weeks, subjects returned to the study site for their follow-up evaluation of malodor after having refrained from brushing for 12 hours (overnight).
- **Results:** Eighty (80) subjects completed the study. After three weeks of product use, subjects in the Dual Zinc plus Arginine dentifrice group and the regular fluoride dentifrice group showed statistically significant ($p < 0.001$) reductions of 38.9% and 11.6%, respectively, in organoleptic scores as compared to baseline. Relative to the regular fluoride dentifrice group, subjects in the Dual Zinc plus Arginine dentifrice group exhibited a statistically significant ($p < 0.001$) reduction of 30.8% in oral malodor. The quality of breath for subjects in the Dual Zinc plus Arginine dentifrice group was in the range corresponding to pleasant breath, whereas the quality of breath for subjects in the regular fluoride dentifrice group was in the range corresponding to unpleasant breath.
- **Conclusion**: The overall results of this double-blind clinical study support the conclusion that a new Dual Zinc plus Arginine dentifrice containing zinc (zinc oxide, zinc citrate) 0.96%, 1.5% arginine, and 1450 ppm fluoride as sodium fluoride in a silica base provides a significantly greater reduction in oral malodor as compared to a regular fluoride dentifrice containing 1450 ppm fluoride as sodium fluoride in a silica base 12 hours post-brushing (overnight) after 3 weeks of product use.

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Introduction

Oral malodor, also known as bad breath or halitosis, is defined as persistent unpleasant odor originating from the oral cavity.[1] Studies[2-7] have shown that up to 50% of the adult population suffers from oral malodor at one point in their life; however, the actual prevalence is uncertain. Halitosis, for many individuals, is the cause of significant psychological discomfort and social embarrassment.

In roughly 75% of the cases, bad breath originates from the mouth itself; however, bad breath can also emanate from systemic conditions such as diabetes or hepatic and renal failure.[8,9] Problems such as deep carious lesions, periodontal or peri-implant disease, oral infections, mucosal ulcerations, impacted food or debris, and tongue coating[8,10] contribute to bad breath, with bacterial metabolism being the root of the problem. Although oral conditions can be the source of malodor, there is significant evidence indicating that the tongue dorsum is a principal source of bad breath.[11-13] The tongue serves as a primary reservoir for gram-negative bacteria, which are mainly responsible for oral malodor formation.[14,15]

Halitosis results from the metabolism of organic substrates by anaerobic bacteria. Gram-negative bacteria produce volatile sulfur compounds[16] (VSCs) such as hydrogen sulfide and methyl mercaptan,[17] which are extremely noxious, even at trace levels. It has been demonstrated that around 85% of bad breath consists of VSCs.[18,19] There are over 700 bacterial species found in the oral cavity, and it is has been shown that at least 82 of them are capable of producing hydrogen sulfide and another 25 are able to generate methyl mercaptan.[1]

Mints, chewing gums, and sprays are used to mask halitosis, but

since they do not treat the cause of the problem their effectiveness is only temporary. Additionally, there are several dentifrices on the market today that claim to treat multiple indications, including bad breath. One approach is incorporating flavors to mask the odors from the VSCs. This approach is best viewed as a transient approach since the volatile flavor component will dissipate and be cleared from the oral cavity. To truly be effective, the source of the odors, the bacteria found on the tongue surface[20] and the resultant VSCs, need to be directly addressed. It is generally agreed that resolving halitosis arising from bacteria should include the reduction of the intraoral bacterial load and/or the conversion of VSCs to nonvolatile substrates.[8]

Since bacteria are the major source of oral malodor, and they often reside in locations that are not routinely brushed during daily oral hygiene, a logical solution to better manage oral malodor would be to use toothpastes containing antibacterial agents that can kill plaque bacteria that are missed during the mechanical removal of plaque bacteria via tooth brushing.[21] Metal ions, such as zinc, are known for their effectiveness in controlling oral malodor because of their antimicrobial activity.[22] In addition to this, zinc ions have a strong affinity for the thiol groups present in VSCs[23] and demonstrate immediate inhibitory effects on VSC production in contrast to other commonly used actives. This is believed to be due to zinc's ability to effectively and directly reduce the volatile nature of VSCs.[24] This second mode of action possessed by zinc ions is not operable in other antimicrobial agents such as triclosan,[25,26] chlorhexidine,[27] or cetylpyridinium chloride.[1] However, antibacterial dentifrices do not generally succeed in completely eliminating oral malodor for two reasons: (1) antibacterial toothpastes focus on cleaning the teeth and leave behind bacteria on the oral soft tissues; and (2) eliminating bacteria is not an immediate strategy for limiting malodor, and requires time to reduce bacteria below the threshold of detectable malodor.

A holistic model of oral health strives not to sterilize the oral cavity, but to establish a balanced microflora that is able to resist the overgrowth of invasive organisms and maintain a low level of immune surveillance that is optimal for health.[28,29] Whole mouth health does not just mean a lack of plaque, but also a reduction of bacteria on the soft tissues. Soft tissue-based organisms are more likely to play a role in malodor, and so a reduction of bacteria on all oral surfaces will have a greater impact on oral malodor than simply a reduction in dental plaque.

The new Colgate® Total® dentifrice, formulated with zinc (zinc oxide, zinc citrate) 0.96%, 1.5% arginine, and 1450 ppm F as NaF in a silica base (Colgate-Palmolive Co., New York, NY, USA), has been shown to effectively control bacteria on all oral surfaces, both hard and soft tissue.[30] It is the goal of this study to assess the efficacy of the new Colgate Total for the management of oral malodor via organoleptic scores 12 hours post-brushing (overnight) after 3 weeks of twice-a-day (morning and evening) product use, in comparison to a regular fluoride toothpaste containing 1450 ppm fluoride ion.

To maintain a healthy mouth, it is important to limit the growth of bacteria as a means of preventing overgrowth and dysbiosis, but some level of bacteria are needed to maintain the general health of the soft tissues. In this context, while large-scale killing of bacteria will limit oral malodor in the short term, a balanced oral flora, coupled with an active ingredient like zinc that is able to neutralize the odor-causing compounds in breath, will provide both short- and long-term maintenance of more pleasant breath. In fact, one of the most exciting aspects to this new technology is the ability for fast neutralization of odors and the shifting of breath quality from "bad" to "good" without compromise.

The study protocol was reviewed and approved by the Chinese Stomatological Association Institutional Review Board (IRB approval #:CSAIRB2015021) in compliance with international regulations.

Materials and Methods

Subjects and Study Design

This clinical study was performed at the West China College of Stomatology, Sichuan University in Sichuan, People's Republic of China. It employed a randomized, double-blind, and parallel-group design. A total of 80 adult male and female subjects were enrolled into the study based upon the following inclusion and exclusion criteria. Subjects were included in the study if they: (i) were between the ages of 18 and 70 (inclusive); in good general health; in good oral health based on self-assessment; possess a minimum of 20 natural uncrowned teeth (excluding third molars); and were available for the three-weeks' duration of the study for all time point assessments, and signed an informed consent form.

Subjects were excluded from the study for the following reasons: (i) full or partial (upper or lower) dentures; (ii) Immunocompromised (HIV, AIDS, immune suppressive drug therapy), medical conditions prohibiting them from not eating or drinking for the post-use treatment evaluation time points (six hours plus overnight), pregnant or breast feeding; (iii) use of tobacco and phenolic-flavored products, such as mint flavored candies or chewing gum, the morning of the study or during the sampling periods; (iv) history of allergies to personal care/consumer products or their ingredients or to common mouthwash ingredients; (v) participating in any other clinical study during the duration of this study.

Qualifying subjects who met the inclusion/exclusion criteria were randomly assigned to one of two treatment groups and provided with their assigned dentifrice and a Colgate adult soft bristle toothbrush for home use. They were instructed to brush twice daily (morning and evening) for one minute with approximately 1.5 grams of their assigned dentifrice for a period of three weeks. After three weeks, subjects returned to the study site for their follow-up evaluation of malodor after having refrained from brushing for 12 hours.

Qualifying subjects and all clinical study site personnel were blinded to product assignment. All dentifrices were covered with white overwrapping in order to conceal product identity. Label information on each tube consisted of a dentifrice code (study group code), instructions for at-home use, and safety information, including emergency contact information. No at-home instructions were provided as to the method of brushing.

Dentifrices Tested

Test Dentifrice: a Dual Zinc plus Arginine dentifrice containing zinc (zinc oxide, zinc citrate) 0.96%, 1.5% arginine, and 1450 ppm F as NaF in a silica base (Colgate-Palmolive Co., New York, NY, USA).
Control Dentifrice: a regular fluoride dentifrice containing 1450 ppm fluoride as NaF in a silica base (Colgate-Palmolive Co., New York, NY, USA).

Clinical Scoring Procedures

Organoleptic Hedonic Odor Ratings. A panel of 4 trained and calibrated odor judges assigned the breath odor scores of each subject. A specially designed screen was used to hide the identities of both the judges and subjects. It only permitted the judges to be exposed to the breath of each individual. When standing in front of this barrier, with the judges on the opposite side, the subjects were instructed to close their mouth, breathe through their nose and not to swallow for 2 minutes. Subjects were then asked to place their mouth over the one end of an autoclaved breathing cylinder and breathe gently. Each judge then placed their nose at the opposite side of the cylinder and assessed oral malodor using the following hedonic scale:[31] 1= Most Pleasant; 2= Very Pleasant; 3= Moderately Pleasant; 4= Slightly Pleasant; 5= Neither Pleasant Nor Unpleasant; 6= Slightly Unpleasant; 7= Moderately Unpleasant; 8= Very Unpleasant; 9= Most Unpleasant. Following individual scoring, an overall score was determined for each subject by averaging the scores assigned by the four judges.

Oral Soft and Hard Tissue Assessment. The dental examiner visually examined the oral cavity and peri-oral area using a dental light and dental mirror. This examination included an assessment of the soft and hard palate, gingival mucosa, buccal mucosa, mucogingival fold areas, tongue, sublingual and submandibular areas, salivary glands, and the tonsillar and pharyngeal areas.

Adverse Events. Adverse events were obtained from an interview with the subject and from an oral examination by dental examiner.

Statistical Methods

The sample size of 80 subjects (40 subjects per group) was determined based on the overnight organoleptic standard deviation between products of 2.5, a significance level of $\alpha = 0.05$, a 10% attrition rate, and an 80% power level, which allowed this study to detect a minimal statistically significant difference between the study groups means of 4.00. The sample size calculation utilized historical data from a previous study.[14,31,32]

For each subject at each evaluation time point, the hedonic breath-odor scores assigned by the four judges were averaged to yield a single subject-wise score. Statistical analyses were performed on these average organoleptic hedonic scores. Comparisons between the treatment groups with respect to gender were performed using a chi-square analysis, and for age using an analysis of variance (ANOVA). Comparisons of the treatment groups with respect to baseline organoleptic scores were performed using an independent t-test. Within-treatment comparisons of the baseline versus follow-up organoleptic scores were performed using paired t-tests. Comparisons of the treatment groups with respect to baseline-adjusted organoleptic scores at the follow-up examinations were performed using analysis of covariance (ANCOVA). All statistical tests of hypotheses were two-sided and employed a level of significance of $\alpha = 0.05$.

Results

All eighty (80) subjects successfully completed the three-week clinical study. There were no adverse events associated with products use observed or reported during the course of the study. The treatment groups did not differ statistically significantly with respect to the gender ($p = 0.263$) or age ($p = 0.968$) characteristics. The gender and age of the study population are presented in Table I.

Table I
Summary of Age and Gender

Treatment	Number of Subjects			Age	
	Male	Female	Total	Mean ± SD	Range
Test Dentifrice[1]	23	17	40	43.13 ± 11.26	20–62
Control Dentifrice[2]	18	22	40	43.23 ± 10.62	20–61

[1]A Dual Zinc plus Arginine dentifrice containing zinc (zinc oxide, zinc citrate) 0.96%, 1.5% arginine and 1450 ppm F as NaF in a silica base (Colgate-Palmolive Co., New York, NY, USA).
[2]A regular fluoride dentifrice containing 1450 ppm fluoride as NaF in a silica base (Colgate-Palmolive Co., New York, NY, USA).

Baseline and Three-Week Data

Table II presents a summary of the organoleptic score means recorded at the baseline examination for those subjects who completed the three-week clinical study. For the organoleptic oral malodor assessments, the mean baseline scores were not statistically different ($p = 0.968$), with 7.35 for subjects assigned to the Test Dentifrice Group and 7.34 for subjects assigned to the Control Dentifrice Group. There were, however, statistical differences when comparing the treatment group to the baseline, as well as to the Control Group.

Relative to the baseline data, the Test Group and the Control Group showed statistically significant ($p < 0.001$) reductions of 38.9% and 11.6%, respectively, in organoleptic scores 12 hours post-brushing (overnight) after three weeks of product use. Additionally, when compared to the Control Group, subjects in the Test Group exhibited a statistically significant ($p < 0.001$) 30.8% improvement in breath quality via organoleptic scores 12 hours post-brushing (overnight) after 3 weeks of product use. After treatment, the quality of breath for subjects in the Test Group was in the range corresponding to

Table II
Organoleptic Scores at Baseline and 12 Hours Post-Brushing (Overnight) After Three Weeks of Products Use

Parameter	Treatment	n	Baseline Mean (±SD)		Adjusted 12-Hour Post-Brushing Mean (±SE)		Within-Treatment Analysis Percent Change[3]	Within-Treatment Analysis Sig.[4]	Between-Treatment Comparison Percent Diff.[5]	Between-Treatment Comparison Sig.[6]
Organoleptic Ratings	Test Dentifrice[1]	40	7.35	0.26	4.49	0.07	38.9%	p <0.001	30.8%	p <0.001
	Control Dentifrice[2]	40	7.34	0.30	6.49	0.07	11.6%	p <0.001		

[1] A Dual Zinc plus Arginine dentifrice containing zinc (zinc oxide, zinc citrate) 0.96%, 1.5% arginine, and 1450 ppm F as NaF in a silica base (Colgate-Palmolive Co., New York, NY, USA).
[2] A regular fluoride dentifrice containing 1450 ppm fluoride as NaF in a silica base (Colgate-Palmolive Co., New York, NY, USA).
[3] Percent change exhibited by the 12-hour post-brushing (overnight) after three weeks of product use mean relative to the baseline mean. A positive value indicates a reduction in organoleptic scores at 12-hour post-brushing (overnight) after three weeks of product use.
[4]Significance of paired t-test comparing the baseline and the 12-hour post-brushing (overnight) after three weeks of product use.
[5] Difference between the 12-hour post-brushing (overnight) after three weeks of product use means expressed as a percentage of the 12-hour post-brushing (overnight) after three weeks of product use mean for the Control Group. A positive value indicates a reduction in organoleptic scores for Test Group relative to the Control Group.
[6] Significance of ANCOVA comparison of baseline-adjusted means.

pleasant breath; in the case of the Control Group, the subjects' breath quality remained in the range corresponding to unpleasant breath.

As shown in Figure 1, 90% of subjects in the Test Group's breath quality transitioned from a range corresponding to unpleasant breath at the beginning of the study to pleasant breath upon completion of the study. This is in contrast to the Control Group in which 95% of the subjects' breath quality remained in the unpleasant range upon completion of the three weeks of product usage.

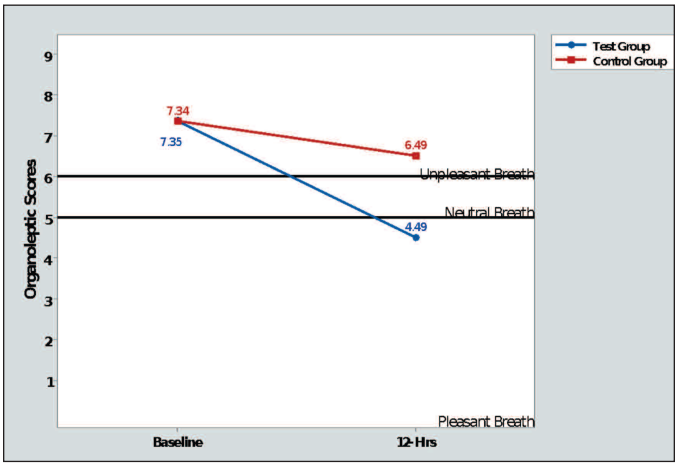

Figure 1. *A graphic overview of subjects' organoleptic scores 12 hours post-brushing (overnight) after three weeks of product use. Test Group: A Dual Zinc plus Arginine dentifrice containing zinc (zinc oxide, zinc citrate) 0.96%, 1.5% arginine, and 1450 ppm F as NaF in a silica base (Colgate-Palmolive Co., New York, NY, USA). Control Group: A regular fluoride dentifrice containing 1450 ppm fluoride as NaF in a silica base (Colgate-Palmolive Co., New York, NY, USA).*

Discussion and Conclusions

In a traditional view, oral malodor has been considered a secondary, cosmetic problem largely left to be treated by covering odors with mints, etc. However, a truly healthy mouth will be resilient to malodor as well. Oral malodor is largely driven by the metabolism of food particles and cellular debris by oral bacteria, leading to the release of VSCs.[16-19] With this understanding, it becomes clear how the addition of an antibacterial agent to a dentifrice would contribute to an overall reduction in oral malodor.

It is well known that zinc, like other similar ions such as copper and tin, can effectively bind to sulfur. The binding of sulfur-containing molecules to zinc results in complexes with very low volatility.[23] The net effect of such binding is a lowering of the disagreeable odor as the VSCs are removed from the gas phase. The metal-sulfur interaction has much higher kinetics and would provide a more rapid improvement in breath quality during the time required for reduced bacterial loads to contribute to overall malodor reductions.

As was presented by Manus[33] *et al.*, the Dual Zinc plus Arginine toothpaste was developed to provide an increased bioavailable amount of zinc. The authors demonstrated that along with increased solubilization there was a concomitant delivery and penetration of zinc to bacteria and biofilm *in vitro.* The delivered zinc resulted in a net decrease of biofilm as measured *in vitro*. The effect was further shown to be translated *in vivo* by Zhang,[34] *et al*. with a significant decrease in oral plaque when subjects brushed with the Dual Zinc plus Arginine toothpaste. Similar to the work in this paper, Fitzgerald,[35] *et al*., have shown through both organoleptic and hedonic measurements a net decrease in VSCs and an overall improvement in breath quality. These effects on oral malodor are not only similar to those shown in this paper but have been demonstrated overnight after a single use.

As described in this Special Issue,[30,33] the test dentifrice in this study is able to promote overall oral health using a whole mouth ecological approach rather than total bacterial kill. This approach hypothesizes that it is not the presence of specific bacteria in the mouth that is solely responsible for disease, but that the balance of bacteria in the mouth is important. This shifts the burden for whole mouth health interventions from a disease prevention approach of sterilizing, as much as possible, oral surfaces, to a means of promoting a balance between bacterial load and its associated conditions, and low level host-driven control of oral bacteria.

Capitalizing on the strong propensity for zinc to bind VSCs, the Dual Zinc plus Arginine dentifrice was confirmed to improve breath after 12 hours. While brushing with a regular fluoride toothpaste can result in improvement, there is further statistical improvement (30.8%) when brushing with this new therapeutic toothpaste. This is a critical cosmetic benefit to consumers who often awaken to a stale or self-perceived bad breath, referred to as "morning mouth." As was shown in Figure 1, the breath quality of the Test Group was able to be statistically shifted after an overnight period of 12 hours to "neutral" when the dentifrice had been previously used for three weeks. This is an important target and point of distinction for this technology. The breath quality was not only able to be altered from unpleasant, but by being neutral the implication is that other residual masking odors are not present. The magnitude of the breath improvement is important to note, as 90% of the Test Group experienced a statistical shift from unpleasant breath to neutral. The significance of this shift is dramatized when compared to the Control Group in which 95% showed no improvement. In essence the offending odors are truly neutralized when using the Dual Zinc plus Arginine toothpaste.

The overall results of this double-blind clinical study support the conclusion that a Dual Zinc plus Arginine dentifrice containing zinc (zinc oxide, zinc citrate) 0.96%, 1.5% arginine, and 1450 ppm fluoride as sodium fluoride in a silica base provides a significantly greater reduction in oral malodor as compared to a dentifrice containing 1450 ppm fluoride as sodium fluoride in a silica base 12 hours post-brushing (overnight) after three weeks of product use.

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Conflict of Interest: Drs. Delgado and Zhang are employed by the Colgate-Palmolive Company. All other authors declare no conflict of interest.

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Short- and Long-Term Effects of a Dentifrice Containing Dual Zinc plus Arginine on Intra-Oral Halitosis: Improvements in Breath Quality

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Abstract

- **Objective:** These studies aimed to assess the short-term (12-hour, single use) and long-term (four weeks, continuous use) efficacy of a new Dual Zinc plus Arginine dentifrice against intra-oral halitosis versus a negative control.
- **Methods:** Two clinical studies were conducted to assess the dentifrice: a four-week, continuous use parallel design versus a negative control and a single use crossover design versus a negative control. Both studies used organoleptic and hedonic odor judge scores measured 12 hours overnight after product use as the primary efficacy variable. Additionally, the single use study employed SIFT-MS to quantify the intra-oral concentration of volatile sulfur compounds as a complementary measure of efficacy.
- **Results:** In both studies, the Dual Zinc plus Arginine dentifrice provided statistically significant improvements in breath quality across all measures versus a negative control.
- **Conclusion:** Improvements in breath quality were attributed to the effects of zinc cations delivered by the uniquely formulated dentifrice.

(J Clin Dent 2018;29(Spec Iss A):A46–54)

Introduction

Halitosis is a common condition that can cause personal discomfort and social embarrassment. While etiology and severity of the condition vary, multiple studies estimate the prevalence of halitosis to be around 25%.[1-3] Halitosis originating from the oral cavity, sometimes referred to as intra-oral halitosis or oral malodor, is the most common manifestation, and it accounts for 80–90% of halitosis cases. Systemic, dietary, and other extra-oral causes account for the remainder.[4]

Volatile compounds arising from bacterial metabolism in the oral cavity, chiefly from anaerobes on the tongue, are the primary cause of intra-oral halitosis. The tongue is anatomically predisposed to harbor abundant numbers of bacteria owing to its large, papillated surface, and its crypts and fissures that provide suitable environments for anaerobes to thrive.[5] The anaerobes decompose proteinaceous components of saliva, desquamated epithelial cells, and trapped foodstuffs transforming them into foul-smelling volatile sulfur compounds (VSCs) and volatile organic compounds (VOCs).[6] The quantity of volatiles produced is closely associated with the population density of bacteria on the tongue.[7,8]

Due to their low odor thresholds and high volatility, VSCs (mainly hydrogen sulfide, methyl mercaptan, and dimethyl disulfide) are the critical effectors of breath odor and intensity in halitosis sufferers.[9] VOCs such as amines, short chain fatty acids, and indoles further shape breath odor, and the specific composition of volatiles in breath can be indicative of differences in microflora composition and activity, and potentially of oral diseases.[10,11] Halitosis is associated with increases in activity and or abundance of certain a bacterial genera such as *Fusobacterium, Porphyromonas, Prevotella,* and *Tanerella* in tongue biofilms.[12,13] Members of those genera are implicated in gum disease, which also shows a positive association with halitosis.[14-16] Furthermore, research suggests that the volatiles themselves can be harmful. For example, the VSCs hydrogen sulfide and methyl mercaptan have been shown to be deleterious to oral soft tissues, as well as the VOCs propanoic and butyric acid.[17-19] Thus, it is thought that breath quality can be indicative of oral health under certain circumstances.

Evaluation of halitosis relies primarily on organoleptic (intensity) and hedonic (pleasant/unpleasant) assessment of breath by trained odor judges, and while they are qualitative methods, they remain the

standard measures used for halitosis research.[20] Quantitative instrumental methods are available, but most instruments are limited by what volatiles can be measured or indirect sampling techniques.[21] Popular instruments include the Halimeter, a sensor-based VSC detector, and the OralChroma, a VSC detector based on gas chromatography. While the Halimeter offers direct measurement of VSCs in breath, it cannot discriminate between specific VSCs. And though the OralChroma can discriminate between VSCs, it cannot sample breath in real time. Samples are first collected in a syringe and then later analyzed. Neither instrument can measure the VOCs mentioned above. Selected ion flow tube–mass spectrometry (SIFT-MS) offers a solution to those limitations.

SIFT-MS is a real-time, quantitative method for analyzing a wide range of volatile compounds. (See Španěl, *et al*. for a detailed treatment of the methodology.[22]) Recent applications of SIFT-MS include the analysis of volatiles in the headspace of microbial cultures and in exhaled human breath, and other gas samples for medical diagnosis and monitoring of treatments.[23,24] Saad, *et al*. have employed SIFT-MS for the analysis of volatiles from *in vitro* biofilm matrices inoculated with human-derived tongue biofilm before and after treating the matrices with bacteria- and odor-reducing compounds.[25] Saad, *et al*. have also used SIFT-MS to measure VSCs and VOCs concentrations of the mouth air of individuals before and after treatment for halitosis (unpublished work).

Treatment of halitosis typically employs fastidious hygiene with floss and brush to remove bacteria from oral surfaces and the use of oral care products containing antimicrobial and odor-neutralizing agents (see reference for an exhaustive list of treatment strategies).[26] Zinc is a widely applied agent due to its joint antimicrobial and odor-neutralizing properties. Cations of zinc exert a bacteriostatic effect thereby reducing production of VSCs and form insoluble complexes with VSCs rendering them odorless.[27] The efficacy of zinc-containing oral care products depends on the bioavailability of the zinc cation. Excipients in a given product formulation can reduce or enhance the bioavailability of zinc cations and therefore affect the efficacy of a product. Certain combinations of zinc sources and amino acids can provide enhanced bioavailability, and lead to formulae with unique performance characteristics where zinc cations are available at both short and long time scales after use.[28]

This work aimed to evaluate the mitigating effects of a dentifrice containing 0.96% zinc (zinc oxide, zinc citrate), 1.5% arginine and 1450 ppm fluoride as sodium fluoride in a silica base (hereon referred to a Dual Zinc plus Arginine) on intra-oral halitosis in both short-term (12 hours overnight after a single use) and long-term (four weeks continuous) usage regimes. Organoleptic and hedonic assessments were used to evaluate improvements in breath quality attributable to the Dual Zinc plus Arginine dentifrice in separate studies for each usage regime. Additionally, SIFT-MS was used to quantify VSC reduction attributable to the Dual Zinc plus Arginine dentifrice in the short-term study.

Materials and Methods

Ethical Approval

Each study protocol was separately reviewed and approved by the Wales REC 6 Committee, UK (ref: 15/WA/0376) and by the Wales Research Ethics Committee 1, UK (ref: 17/WA/0170) respectively, and by the internal committees of Colgate-Palmolive and the University of the West of England.

Study Design

The first study (four-week, long-term use) was a four-cell, double-blinded parallel study, which involved the recruitment of 150 individuals who were screened for intra-oral malodor. The cells comprised the test product (Dual Zinc plus Arginine), a matching placebo (negative control), and two experimental products that are not described in this paper. Out of 150 volunteers, 130 fulfilled the inclusion criteria and 126 were retained, comprised of 52 male (mean age 36.7) and 74 female (mean age 35) volunteers. Eligibility criteria included age between 18 and 65 years, medium to high malodor with an organoleptic score between 2 and 5 on the organoleptic scale and 0 to -5 on the hedonic scale, and their availability at the study site at different sampling times and days, with no history of allergy to personal care consumer products or their ingredients, and having given informed consent. Exclusion criteria included moderate to advanced periodontal disease or untreated caries (as determined by a dentist), and hard and soft tissue diseases, substantial false dentition, xerostomia, use of antibiotics or antimicrobial drugs within 30 days prior to the study, consumption of medicated sweets containing antimicrobial agents at least one week before and during trial, medical history of infectious disease (HIV, hepatitis, tuberculosis), pregnant or nursing women, consumption of foods associated with oral malodor (*e.g*., garlic, onions, spices) on the day prior to, and on sampling day, and wearing of strongly perfumed cosmetics on sampling day.

The second study (12-hour overnight, single product use) consisted of a randomized, double-blinded, two-way crossover clinical study comprising two cells: the test product (Dual Zinc plus Arginine) and a negative control. A group of 70 volunteers from the University of the West of England, aged between 18 and 65 years, were screened for intra-oral malodor, were enrolled and fulfilled the inclusion criteria of which 64 completed the study. They were 24 males (mean age 35.2) and 40 females (mean age 37.6), respectively. The same inclusion and exclusion criteria were applied as in the first study. The breath freshening effect of a zinc-containing toothpaste against a negative control was assessed 12 hours overnight after a single brushing.

Clinical Procedures

In the first study (four-week continuous use, parallel), 150 participants were recruited of which 126 completed the study. On the first visit following baseline measurements, all participants were given a toothbrush (Colgate® Slim Soft, Colgate-Palmolive Company, New York, NY, USA) and a washout toothpaste (Colgate® Cavity Protection, Colgate-Palmolive Company, New York, NY, USA) to be used twice a day for one week prior to treatment. A total of 33 participants were assigned to the treatment toothpaste (Dual Zinc plus Arginine) and another 33 to the negative control, a matching placebo toothpaste containing 1450 ppm F as NaF. Both treatment and negative control were wrapped with a plain white label marked as treatment A or B; therefore the study was double-blinded. All volunteers used one treatment for four weeks, with a one-week washout period prior to treatment. All participants were provided with their information letter, protocol, diary and appointment dates/times for attending the laboratory. With the

exception of the four monitoring mornings, subjects were not asked to alter their normal oral hygiene regime throughout the four-week study. Participants were advised to use the test toothpaste twice a day for four weeks and brush their teeth for at least two minutes. Prior to the evaluation days (night before), volunteers were advised to continue their normal oral hygiene habit but to avoid oral hygiene (brushing their teeth) and food intake on the mornings of their overnight assessments. Subjects were evaluated at baseline, one week, two weeks, and four weeks.

For the second crossover study (12-hour overnight single product use, crossover), all participants who fulfilled the inclusion criteria were randomly assigned a number from 1 to 70. On the first visit and following baseline measurements, all participants were given a toothbrush (Colgate Slim Soft) and a washout toothpaste (Colgate Cavity Protection) to be used twice a day for one week prior to treatment. After completing the washout period, the participants presented to the laboratory and mouth air analysis was performed; based on the criteria set up above, 64 participants were retained. These participants were randomized, divided into two groups of 32 each, and assigned a treatment-use sequence. The same number of sequences were equally represented in each group. However, only 61 participants were able to complete the study. Dual Zinc plus Arginine was used as the test paste and the washout toothpaste (Colgate Cavity Protection) was used as the negative control. Both treatment and negative control were wrapped with a white label marked as treatment A or B; therefore the study was double-blinded. Following baseline measurements, participants were instructed to brush their teeth with the washout toothpaste and to use the first treatment toothpaste once in the evening, 12 hours before sampling the following morning. It was also emphasized to participants that tooth brushing should be performed for precisely 2 minutes using the allocated treatment and soft-bristled toothbrush supplied. The following morning, participants returned to the laboratory without brushing their teeth and without taking breakfast, for mouth air sampling and analysis by organoleptic, hedonic, and SIFT-MS measurement of VSCs. They were then instructed to use the washout toothpaste for another week. On the third visit, they reported to the laboratory for another baseline measurement of their breath and were provided with the second treatment which they could only use 12 hours before their second treatment/control, in the same manner as for the first treatment. The participants returned to the laboratory the following morning for their final breath analysis, thus completing the study.

Organoleptic and Hedonic Odor Judge Score

In both studies, the quality of individual's mouth air was evaluated prior to and following treatment. Upon arrival, each participant had their breath assessed by a trained organoleptic judge using the six-point organoleptic scale (0 to 5) as outlined by Rosenberg and McCulloch,[20] but modified in terms of odor descriptions according to Greenman, *et al*. as follows: 0 = no odor, 1 = barely noticeable odor, 2 = slight odor, 3 = moderate odor; 4 = strong odor, and 5 = extremely strong odor close to saturation.[10,16] The odor judge assessed the pleasantness or unpleasantness of the breath on a 10-point hedonic scale according to ASTM 1968b where zero to minus five (0 to -5) corresponds to the degree of unpleasantness and zero to five (0 to 5) to the pleasantness.[29]

Instrumental Analysis of Mouth Air – SIFT-MS

In the second study, the gas phase analysis of VSCs was performed prior to, and after 12-hours following use of test toothpaste, using a Profile 3 SIFT-MS instrument, UK.[30] The SIFT-MS was used in selected ion mode with the compounds to be investigated [H_2S, CH_3SH, and $(CH_3)_2S$] selected from the drop down library list. For gas analysis, the subjects were instructed to close their mouth for two minutes and breathe through their nose prior to sampling by the SIFT-MS. During this period, approximately one minute of background air levels were measured prior to sampling. After two minutes, the subjects were instructed to place a clean sampling straw into their mouth, to place their teeth and lips gently against the straw, without compressing or restricting it, so that the tip of the straw is situated over the back of their tongue without touching the inside of their mouth. The subjects were also instructed to keep the muscles around the mouth relaxed and breathe gently through their nose during the sampling. Approximately one minute of data was recorded, and about one minute of background levels were measured post sampling. Resultant data were saved and then analyzed using the SIFT-MS profile viewer software. The first 40 seconds of data following the plateau of the water concentration value was used to give the gas concentrations of the compounds investigated. If significant non-zero levels of any of the compounds in the background air were found, these values would be subtracted from the sample values. The quality of the sample is determined by a concentration of water in the sample which should be in the range 5.2-6%.[26]

Statistical Analysis

With regard to the four-week study, analysis of covariance (ANCOVA) was used to test for significant differences among treatment groups. The ANCOVA model included a treatment effect as well as the baseline response as a covariate. A treatment effect p-value of < 0.05 was used to indicate statistical significance. A Tukey multiple comparison test was used to assess pair-wise differences among the treatment groups. A separate analysis was completed for one, two, and four weeks. As to the crossover study, data were analyzed using analysis of variance (ANOVA). The model included effects for treatment, period, sequence, and subject. The baseline malodor level was included in the model as a covariate. A Tukey pairwise test was used to assess differences between the treatment groups for the organoleptic and hedonic scores. A one-sided Dunnett's test was used to assess differences between the treatment groups for the VSC measurements. A p-value of < 0.05 was used to indicate statistically significant effects.

Results

Study 1: Four-Week, Long-Term Usage

Figure 1 shows the organoleptic odor judge scores from the first study. The test product effected a statistically significant reduction in organoleptic scores (a reduction in breath odor intensity) after one week of use compared to baseline scores (3.09 to 2.53, $p < 0.05$), and continued to reduce scores in weeks two (to 2.28, $p < 0.05$) and four (to 2.19, $p < 0.05$). Similarly, in Figure 2, the test product produced a statistically significant improvement in hedonic scores (a change from unpleasant to pleasant) after one week of use compared to baseline scores (-1.00 to 0.373, $p < 0.05$), and continued to improve

scores in weeks two (to 0.842, $p < 0.05$) and four (to 1.25, $p < 0.05$). The negative control failed to effect any statistically significant changes from baseline on either measure. At all time points except baseline, the test product scores were statistically significantly better than the negative control scores. (See Appendices A1-A6 for summary study statistics.)

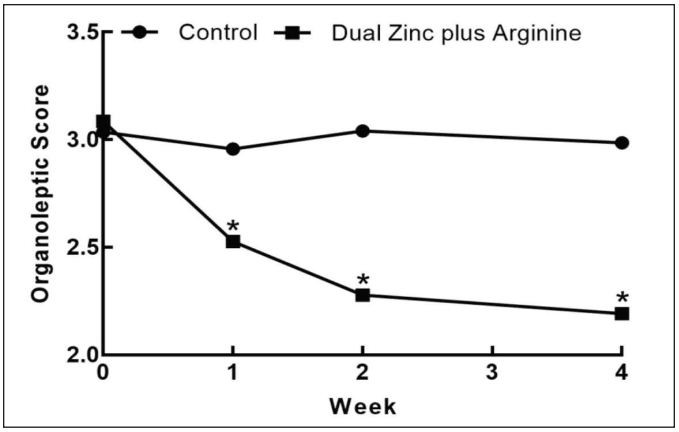

Figure 1. *Adjusted mean 12-hour overnight organoleptic scores at baseline (week 0) then one week, two weeks, and four weeks following test and negative control treatment for each group. Larger values indicate more intense, more noticeable breath odor. Significant difference from baseline (within-group) and treatments are indicated by an asterisk ($p < 0.05$).*

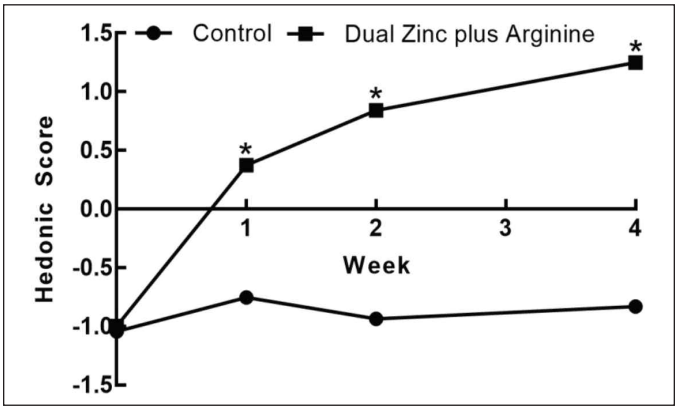

Figure 2. *Adjusted mean 12-hour overnight hedonic scores at baseline (week 0) then one week, two weeks and four weeks following test and placebo treatment for each group. Values greater than zero correspond to neutral/pleasant breath. Values less than zero correspond to unpleasant breath. Significant difference from baseline (within-group) and treatments are indicated by an asterisk ($p < 0.05$).*

Study 2: 12-hour Overnight, Single Use

For the second study it can be seen that the test product effected a statistically significant ($p < 0.05$) freshening effect reflected in both organoleptic and hedonic scores versus the negative control 12 hours after a single brushing. The adjusted mean organoleptic scores (Figure 3) for the zinc-containing paste and the negative control were 2.3 and 3.0, respectively. This represents a 23.3% reduction in odor intensity score versus the negative control. The adjusted mean hedonic scores (Figure 4) for the zinc-containing paste and the negative control were 0.22 and -1.6, respectively, where negative scores indicate unpleasant odor and positive scores indicate neutral to pleasant odor. This represents a scaled* improvement of 54.4% in hedonic score versus the negative control. Raw mean baseline and post-use scores are shown in Tables I and II. These results indicate the capacity of the zinc-containing paste to significantly freshen breath for 12 hours overnight after a single brushing.

**The hedonic scale used during the study ranged from -5 to 5. 5 was added to the mean scores to move the range from 0 to 10 so that an appropriate percent change could be calculated.*

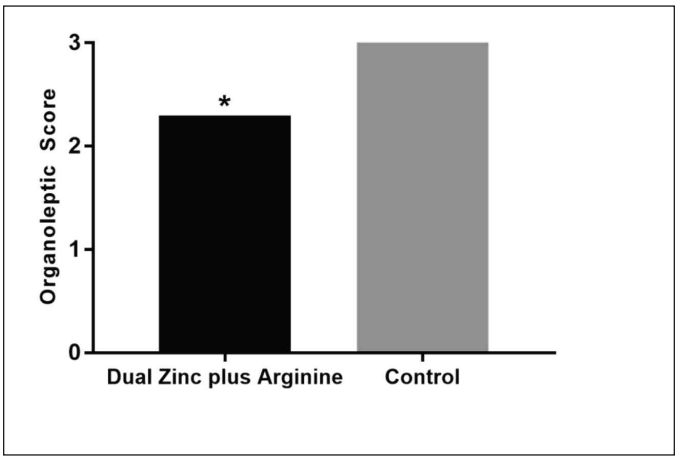

Figure 3. *Mean 12-hour overnight organoleptic scores following a single brushing with the test product or negative control. Significant difference between treatments is indicated by an asterisk ($p < 0.05$).*

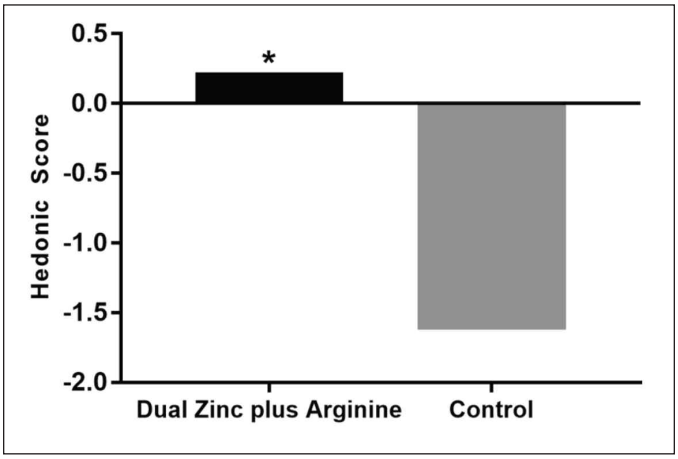

Figure 4. *Mean 12-hour overnight hedonic scores following a single brushing with the test product or negative control. Significant difference between treatments is indicated by an asterisk ($p < 0.05$).*

Table I
12-Hour Overnight, Single Use: Raw Mean Hedonic Scores

Sequence	Baseline 1	Post-Use	Baseline 2	Post-Use
Dual Zinc plus Arginine followed by Control	-2.0	0.13	-1.4	-1.4
Control followed by Dual Zinc plus Arginine	-2.0	-1.7	-1.9	0.19

Table II
12-Hour Overnight, Single Use: Raw Mean Organoleptic Scores

Sequence	Baseline 1	Post-Use	Baseline 2	Post-Use
Dual Zinc plus Arginine followed by Control	3.2	2.3	3.1	3.0
Control followed by Dual Zinc plus Arginine	3.1	3.0	3.0	2.3

The concentration of intra-oral VSCs measured by SIFT-MS (Figure 5) were statistically significantly ($p < 0.05$) reduced by the test product versus the negative control. The composite VSC

concentration is computed as the sums of the natural logs of hydrogen sulfide, methyl mercaptan, and dimethyl disulfide concentrations measured as ppb for each subject. (See Appendices A7-A9 for summary statistics.)

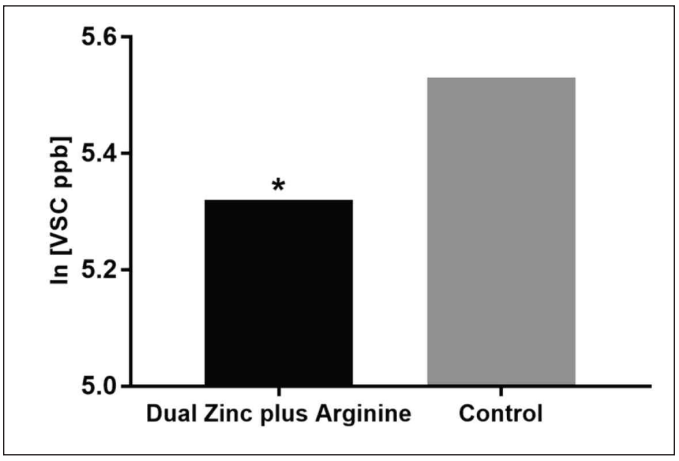

Figure 5. *Concentration of VSCs in oral cavity measured with SIFT-MS 12 hours overnight following a single brushing with the test product or negative control. Reported baseline-adjusted means are of composite concentrations (natural log of the sum of hydrogen sulfide, methyl mercaptan, and dimethyl disulfide concentrations measured as ppb for each subject). Significant difference between treatments is indicated by an asterisk ($p < 0.05$) using a Dunnett* post-hoc *comparison.*

Discussion

Zinc is added to toothpastes and mouth rinses as an antimicrobial and odor-neutralizing agent to fight plaque bacteria (including those that produce malodor) and to neutralize the VSCs that are largely responsible for oral malodor. To exert its anti-halitosis efficacy, zinc cations must be present at the site of malodor production, *e.g.*, tongue biofilms, at an effective concentration for sufficient time. Zinc can be retained in the oral cavity after tooth brushing by binding to protein surfaces on the oral mucosa, in the saliva, or on bacterial surfaces.[31]

However, zinc salts can be difficult to formulate depending on their solubility and the presence of other dentifrice formula ingredients, *e.g.*, phosphates, which may limit the availability of zinc cations. The Dual Zinc plus Arginine dentifrice tested in these studies was specifically designed to overcome those limitations. The inclusion of arginine into the toothpaste increases and preserves the availability of zinc cations helping to deliver an effective concentration to target surfaces in the oral cavity. This can improve the antimicrobial and VSC-neutralizing efficacy of zinc-based dentifrices. Moreover, formulating with two zinc sources enables immediate and sustained reservoirs of zinc cations: particulate zinc oxide supplies a long-lasting reservoir of zinc cations while solubilized zinc citrate provides zinc cations immediately available upon brushing.[28] This is consistent with the observed short- and long-term breath quality improvements.

The two clinical studies in this work compared the efficacy of a Dual Zinc plus Arginine dentifrice for treating intra-oral halitosis in both long-term (four-week, twice-daily brushing) and a short-term (single brushing, 12-hour overnight) usage regimes. In both cases, the test dentifrice significantly improved breath quality with respect to both organoleptic and hedonic scores compared to a negative control. With regard to the single brushing study, VSC levels as measured by SIFT-MS were also significantly reduced, thus demonstrating the short-term odor-neutralizing capacity of available zinc cations delivered by the formula.

The improvement in breath quality after four weeks can be visualized using a breath quality map (Figure 6). The map plots the organoleptic and hedonic scores of subjects at baseline and after four

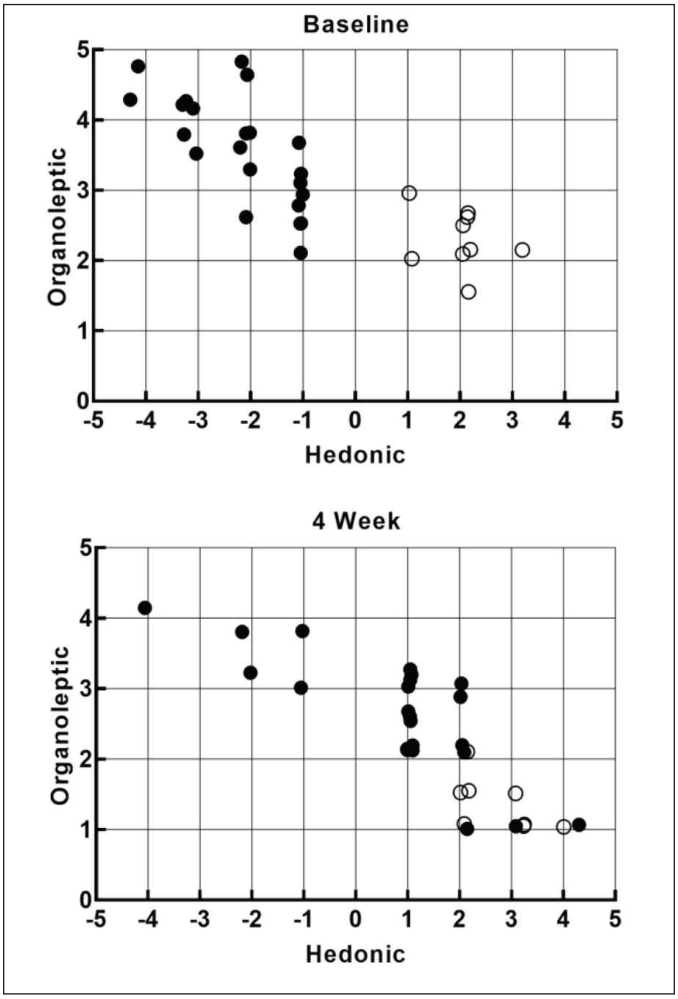

Figure 6. *Breath quality maps of subjects at baseline and four weeks 12 hours overnight after using the Dual Zinc plus Arginine treatment. Agglomerative clustering of organoleptic and hedonic odor judge scores (Euclidian distance, complete linkage) identified two distinct breath types: lower quality breath (●), which is marked by oganoleptic scores > 2 and hedonic scores < 0, and higher quality breath (○), which is marked by organoleptic scores < 3 and hedonic scores > 0. Note that data points were jittered on both axes with approximately 10% relative Gaussian noise to improve legibility.*

weeks of using the Dual Zinc plus Arginine formula. The subjects were grouped into two breath quality types based on agglomerative clustering of their baseline scores. The black circles represent a lower quality breath group, whereas the open circles represent a higher quality breath group (oganoleptic scores > 2 and hedonic scores < 0, and organoleptic scores < 3 and hedonic scores > 0, respectively). The change of position on the map towards the lower right quadrant reflects an improvement in breath quality. The group with the lower quality breath type makes a concerted migration into the higher quality group position, indicating the improvement in breath quality imparted by the Dual Zinc plus Arginine formula.

The SIFT-MS technique proved to be a useful complementary methodology in the single brushing study, allowing for immediate measurement of breath VSC components. The improvement in breath quality in the single brushing study is primarily due to the reduction in VSCs as can be seen in Figure 7. The Dual Zinc plus Arginine formula

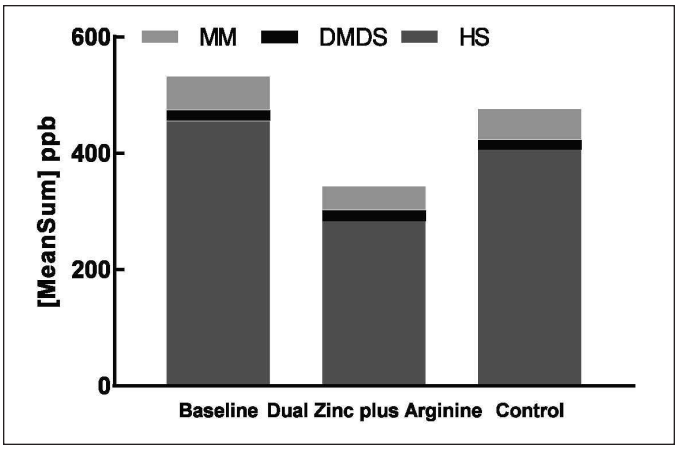

Figure 7. *VSC composition of breath from subjects in 12-hour overnight, single-use study. Concentrations are expressed as raw means of volatile concentration as measured by SIFT-MS.*

reduces both the hydrogen sulfide and methyl mercaptan concentration in mouth air. The relative compositions of the VSCs remain similar suggesting that they are affected equally by the dentifrice. This effect in part is due to the zinc cations in the Dual Zinc plus Arginine formula chemically neutralizing the VSCs. Antimicrobial activity is in part responsible as well. Scavenging VSCs may also provide the additional benefit of protecting the soft tissue from harm done by volatiles like hydrogen sulfide and methyl mercaptan leading to potential improvements in oral health.

Taken together, these results suggest that the Dual Zinc plus Arginine dentifrice can provide short- and long-term improvement in breath quality due to its unique formulation characteristics. The dual zinc source acts for immediate mitigation of intra-oral halitosis and for long-term sustained improvements. These improvements in breath quality can be linked to improvements in oral health by way of the connections between breath odor, the oral microbiome, and gum disease.

Appendices

4-Week Parallel Continuous Use Study – Organoleptic Scores

A1

Parameter	Treatment	n	Baseline (Mean ± S.E.)			Adj. 12 Hour Post-Brushing Mean (S.E.)			Within-Treatment Analysis		Between-Treatment Comparison	
									Percent Change (3)	Sig. (4)	Percent Difference (5)	Sig. (6)
Organoleptic Ratings After 1 Week of 2x Daily Use	Dual Zinc plus Arg (1)	32	3.086	±	0.153	2.527	±	0.166	18.11%	p<0.001	14.54%	p<0.001
	Control (2)	28	3.036	±	0.124	2.957	±	0.132	2.60%	p=0.167		

1.Dentifrice containing Dual Zinc plus Arginine(Colgate-Palmolive., Co, New York,NY).

2. Commercially available fluoride toothpaste (Colgate-Palmolive Co., New York, NY).

3. Percent change exhibited by the 12 hour post-brushing (overnight) after 3 weeks of product use mean relative to the baseline mean. A positive value indicates a reduction in organoleptic scores at 12 hour post-brushing (overnight) after 3 weeks of product use.

4. Significance of paired t-test comparing the baseline and the 12 hour post-brushing (overnight) after 3 weeks of product use.

5. Difference between the 12 hour post-brushing (overnight) after 3 weeks of product use means expressed as a percentage of the 12 hour post-brushing (overnight) after 3 weeks of product use mean for the Control Group. A positive value indicates a reduction in organoleptic scores for Test Group relative to the Control Group.

6. Significance of ANCOVA comparison of baseline-adjusted means.

A2

Parameter	Treatment	n	Baseline (Mean ± S.E.)			Adj. 12 Hour Post-Brushing Mean (S.E.)			Within-Treatment Analysis		Between-Treatment Comparison	
									Percent Change (3)	Sig. (4)	Percent Difference (5)	Sig. (6)
Organoleptic Ratings After 2 Weeks of 2x Daily Use	Dual Zinc plus Arg (1)	32	3.086	±	0.153	2.278	±	0.162	26.18%	p<0.001	25.07%	p<0.001
	Control (2)	28	3.036	±	0.124	3.04	±	0.135	-0.13%	p=0.841		

1.Dentifrice containing Dual Zinc plus Arginine(Colgate-Palmolive., Co, New York,NY).

2. Commercially available fluoride toothpaste (Colgate-Palmolive Co., New York, NY).

3. Percent change exhibited by the 12 hour post-brushing (overnight) after 3 weeks of product use mean relative to the baseline mean. A positive value indicates a reduction in organoleptic scores at 12 hour post-brushing (overnight) after 3 weeks of product use.

4. Significance of paired t-test comparing the baseline and the 12 hour post-brushing (overnight) after 3 weeks of product use.

5. Difference between the 12 hour post-brushing (overnight) after 3 weeks of product use means expressed as a percentage of the 12 hour post-brushing (overnight) after 3 weeks of product use mean for the Control Group. A positive value indicates a reduction in organoleptic scores for Test Group relative to the Control Group.

6. Significance of ANCOVA comparison of baseline-adjusted means.

A3

Parameter	Treatment	n	Baseline (Mean ± S.E.)			Adj. 12 Hour Post-Brushing Mean (S.E.)			Within-Treatment Analysis		Between-Treatment Comparison	
									Percent Change (3)	Sig. (4)	Percent Difference (5)	Sig. (6)
Organoleptic Ratings After 4 Weeks of 2x Daily Use	Dual Zinc plus Arg (1)	32	3.086	±	0.153	2.192	±	0.156	28.97%	p<0.001	30.80%	p<0.001
	Control (2)	28	3.036	±	0.124	2.986	±	0.116	1.65%	p=0.409		

1.Dentifrice containing Dual Zinc plus Arginine(Colgate-Palmolive., Co, New York,NY).

2. Commercially available fluoride toothpaste (Colgate-Palmolive Co., New York, NY).

3. Percent change exhibited by the 12 hour post-brushing (overnight) after 3 weeks of product use mean relative to the baseline mean. A positive value indicates a reduction in organoleptic scores at 12 hour post-brushing (overnight) after 3 weeks of product use.

4. Significance of paired t-test comparing the baseline and the 12 hour post-brushing (overnight) after 3 weeks of product use.

5. Difference between the 12 hour post-brushing (overnight) after 3 weeks of product use means expressed as a percentage of the 12 hour post-brushing (overnight) after 3 weeks of product use mean for the Control Group. A positive value indicates a reduction in organoleptic scores for Test Group relative to the Control Group.

6. Significance of ANCOVA comparison of baseline-adjusted means.

4-Week Parallel Continuous Use Study – Hedonic Scores

A4

Parameter	Treatment	n	Baseline (Mean ± S.E.)			Adj. 12 Hour Post-Brushing Mean (S.E.)			Within-Treatment Analysis		Between-Treatment Comparison	
									Percent Change (3)	Sig. (4)	Percent Difference (5)	Sig. (6)
Hedonic Ratings After 1 Week of 2x Daily Use	Dual Zinc plus Arg (1)	32	-1.000	±	0.365	0.373	±	0.372	137.30%	p<0.001	149.47%	p=0.003
	Control (2)	28	-1.045	±	0.354	-0.754	±	0.314	27.85%	p=0.259		

1.Dentifrice containing Dual Zinc plus Arginine(Colgate-Palmolive., Co, New York,NY).

2. Commercially available fluoride toothpaste (Colgate-Palmolive Co., New York, NY).

3. Percent change exhibited by the 12 hour post-brushing (overnight) after 3 weeks of product use mean relative to the baseline mean. A positive value indicates a reduction in organoleptic scores at 12 hour post-brushing (overnight) after 3 weeks of product use.

4. Significance of paired t-test comparing the baseline and the 12 hour post-brushing (overnight) after 3 weeks of product use.

5. Difference between the 12 hour post-brushing (overnight) after 3 weeks of product use means expressed as a percentage of the 12 hour post-brushing (overnight) after 3 weeks of product use mean for the Control Group. A positive value indicates a reduction in organoleptic scores for Test Group relative to the Control Group.

6. Significance of ANCOVA comparison of baseline-adjusted means.

A5

Parameter	Treatment	n	Baseline (Mean ± S.E.)			Adj. 12 Hour Post-Brushing Mean (S.E.)			Within-Treatment Analysis		Between-Treatment Comparison	
									Percent Change (3)	Sig. (4)	Percent Difference (5)	Sig. (6)
Hedonic Ratings After 2 Weeks of 2x Daily Use	Dual Zinc plus Arg (1)	32	-1.000	±	0.365	0.842	±	0.336	184.20%	p<0.001	190.05%	p<0.001
	Control (2)	28	-1.045	±	0.354	-0.935	±	0.315	10.53%	p=0.744		

1.Dentifrice containing Dual Zinc plus Arginine(Colgate-Palmolive., Co, New York,NY).

2. Commercially available fluoride toothpaste (Colgate-Palmolive Co., New York, NY).

3. Percent change exhibited by the 12 hour post-brushing (overnight) after 3 weeks of product use mean relative to the baseline mean. A positive value indicates a reduction in organoleptic scores at 12 hour post-brushing (overnight) after 3 weeks of product use.

4. Significance of paired t-test comparing the baseline and the 12 hour post-brushing (overnight) after 3 weeks of product use.

5. Difference between the 12 hour post-brushing (overnight) after 3 weeks of product use means expressed as a percentage of the 12 hour post-brushing (overnight) after 3 weeks of product use mean for the Control Group. A positive value indicates a reduction in organoleptic scores for Test Group relative to the Control Group.

6. Significance of ANCOVA comparison of baseline-adjusted means.

A6

Parameter	Treatment	n	Baseline (Mean ± S.E.)			Adj. 12 Hour Post-Brushing Mean (S.E.)			Within-Treatment Analysis		Between-Treatment Comparison	
									Percent Change (3)	Sig. (4)	Percent Difference (5)	Sig. (6)
Hedonic Ratings After 4 Weeks of 2x Daily Use	Dual Zinc plus Arg (1)	32	-1.000	±	0.365	1.249	±	0.305	224.90%	p<0.001	250.12%	p<0.001
	Control (2)	28	-1.045	±	0.354	-0.832	±	0.294	20.38%	p=0.515		

1.Dentifrice containing Dual Zinc plus Arginine(Colgate-Palmolive., Co, New York,NY).

2. Commercially available fluoride toothpaste (Colgate-Palmolive Co., New York, NY).

3. Percent change exhibited by the 12 hour post-brushing (overnight) after 3 weeks of product use mean relative to the baseline mean. A positive value indicates a reduction in organoleptic scores at 12 hour post-brushing (overnight) after 3 weeks of product use.

4. Significance of paired t-test comparing the baseline and the 12 hour post-brushing (overnight) after 3 weeks of product use.

5. Difference between the 12 hour post-brushing (overnight) after 3 weeks of product use means expressed as a percentage of the 12 hour post-brushing (overnight) after 3 weeks of product use mean for the Control Group. A positive value indicates a reduction in organoleptic scores for Test Group relative to the Control Group.

6. Significance of ANCOVA comparison of baseline-adjusted means.

12-Hour Overnight Single Use Crossover Study – Organoleptic, Hedonic, and VSC Scores

A7

Parameter	Treatment	n	Baseline (Mean ± S.E.)			Adj. 12 Hour Post-Brushing Mean (S.E.)			Within-Treatment Analysis		Between-Treatment Comparison	
									Percent Change (3)	Sig. (4)	Percent Difference (5)	Sig. (6)
Organoleptic Ratings 12 Hours, Overnight After Single Use	Dual Zinc plus Arg (1)	61	3.131	±	0.077	2.297	±	0.0795	26.64%	p<0.001	23.56%	p<0.001
	Control (2)	61	3.139	±	0.067	3.005	±	0.0701	4.27%	p<0.05		

1. Dentifrice containing Dual Zinc plus Arginine(Colgate-Palmolive., Co, New York,NY).

2. Commercially available fluoride toothpaste (Colgate-Palmolive Co., New York, NY).

3. Percent change exhibited by the 12 hour post-brushing (overnight) after 3 weeks of product use mean relative to the baseline mean. A positive value indicates a reduction in organoleptic scores at 12 hour post-brushing (overnight) after 3 weeks of product use.

4. Significance of paired t-test comparing the baseline and the 12 hour post-brushing (overnight) after 3 weeks of product use.

5. Difference between the 12 hour post-brushing (overnight) after 3 weeks of product use means expressed as a percentage of the 12 hour post-brushing (overnight) after 3 weeks of product use mean for the Control Group. A positive value indicates a reduction in organoleptic scores for Test Group relative to the Control Group.

6. Significance of ANOVA comparison of baseline-adjusted means.

A8

Parameter	Treatment	n	Baseline (Mean ± S.E.)			Adj. 12 Hour Post-Brushing Mean (S.E.)			Within-Treatment Analysis		Between-Treatment Comparison	
									Percent Change (3)	Sig. (4)	Percent Difference (5)	Sig. (6)
Hedonic Ratings After 2 Weeks of 2x Daily Use	Dual Zinc plus Arg (1)	32	-1.000	±	0.365	0.842	±	0.336	184.20%	p<0.001	190.05%	p<0.001
	Control (2)	28	-1.045	±	0.354	-0.935	±	0.315	10.53%	p=0.744		

1.Dentifrice containing Dual Zinc plus Arginine(Colgate-Palmolive., Co, New York,NY).

2. Commercially available fluoride toothpaste (Colgate-Palmolive Co., New York, NY).

3. Percent change exhibited by the 12 hour post-brushing (overnight) after 3 weeks of product use mean relative to the baseline mean. A positive value indicates a reduction in organoleptic scores at 12 hour post-brushing (overnight) after 3 weeks of product use.

4. Significance of paired t-test comparing the baseline and the 12 hour post-brushing (overnight) after 3 weeks of product use.

5. Difference between the 12 hour post-brushing (overnight) after 3 weeks of product use means expressed as a percentage of the 12 hour post-brushing (overnight) after 3 weeks of product use mean for the Control Group. A positive value indicates a reduction in organoleptic scores for Test Group relative to the Control Group.

6. Significance of ANCOVA comparison of baseline-adjusted means.

A9

Parameter	Treatment	n	Baseline (Mean ± S.E.)			Adj. 12 Hour Post-Brushing Mean (S.E.)			Within-Treatment Analysis		Between-Treatment Comparison	
									Percent Change (3)	Sig. (4)	Percent Difference (5)	Sig. (6)
Hedonic Ratings After 4 Weeks of 2x Daily Use	Dual Zinc plus Arg (1)	32	-1.000	±	0.365	1.249	±	0.305	224.90%	p<0.001	250.12%	p<0.001
	Control (2)	28	-1.045	±	0.354	-0.832	±	0.294	20.38%	p=0.515		

1.Dentifrice containing Dual Zinc plus Arginine(Colgate-Palmolive., Co, New York,NY).

2. Commercially available fluoride toothpaste (Colgate-Palmolive Co., New York, NY).

3. Percent change exhibited by the 12 hour post-brushing (overnight) after 3 weeks of product use mean relative to the baseline mean. A positive value indicates a reduction in organoleptic scores at 12 hour post-brushing (overnight) after 3 weeks of product use.

4. Significance of paired t-test comparing the baseline and the 12 hour post-brushing (overnight) after 3 weeks of product use.

5. Difference between the 12 hour post-brushing (overnight) after 3 weeks of product use means expressed as a percentage of the 12 hour post-brushing (overnight) after 3 weeks of product use mean for the Control Group. A positive value indicates a reduction in organoleptic scores for Test Group relative to the Control Group.

6. Significance of ANCOVA comparison of baseline-adjusted means.

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Conflict of Interest: Michael Fitzgerald, Mark Vandeven, Harsh M. Trivedi, and James G. Masters are currently employees of the Colgate-Palmolive Co. Saliha Saad, Keith Hewett, and John Greenman are collaborative researchers at the University of West England. There is no conflict of interest in this work.

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