OFFICIAL TITLE OF STUDY: A VISUAL EVALUATION OF ORAL PLAQUE REMOVAL UTILIZING AN ADJUNCT ENZYME PRE-RINSE IN ORTHODONTIC SUBJECTS

NCT NUMBER: N/A

DATE: MAY 29, 2018

INDIANA UNIVERSITY

OFFICE OF THE VICE PRESIDENT FOR RESEARCH

Office of Research Compliance

To: Kelton Stewart
DENTISTRY-ORTHO & ORAL FACIAL

Frank Lippert
CARIOLOGY/OPERATIVE&DENTAL PUBLIC HEALTH

Jennifer Rose
UNIVERSITY LEVEL

From:

Chair - IRB-04
Human Subjects Office
Office of Research Compliance – Indiana University

Date: May 29, 2018

RE: **NOTICE OF APPROVAL - NEW STUDY**

Protocol Title: A visual comparison of oral plaque removal in the orthodontic patient utilizing an oral rinse with and without proteolytic enzyme prior to dental brushing and subsequent pulsating water jet cleansing

Study #: 1802369383

Funding Agency/Sponsor: DENTISTRY-ORTHO & ORAL FACIAL

Review Level: Response

Study Approval Date: May 29, 2018

Study Expiration Date: May 14, 2019

The Indiana University Institutional Review Board (IRB) IRB00000219 | IRB-04 recently took action on the above-referenced protocol. The IRB has subsequently verified that the investigator's response to the review satisfies the conditions for approval. In compliance with (as applicable) 21 C.F.R. § 56.109 (e) and 46 C.F.R. § 46.109 (d), this letter serves as written notification of the IRB's determination.

The Board made the following determination(s), as applicable:

- **Plan for soliciting the assent of the children and/or the parent/guardian permission is appropriate.**
- **Child Category 3: greater than minimal risk and no prospect of direct benefit to individual child-subject, but likely to yield generalizable knowledge about the child-subject's disorder or condition.**

Approval of this study is based on your agreement to abide by the policies and procedures of the Indiana University Human Research Protection Program and does not replace any other approvals that may be required. Relevant policies and procedures governing Human Subject Research can be found at: http://researchcompliance.iu.edu/hso/hs_guidance.html.

As a reminder, IRB approval is required prior to implementing any changes or amendments in the protocol, regardless of how minor, except to eliminate immediate hazards to subjects. No changes to the informed consent document may be made without prior IRB approval.

If you submitted and/or are required to provide participants with an informed consent document, **a copy of the most recently approved stamped document is enclosed and must be used to enroll participants.**

The study expiration date is noted above. Failure to receive notification from the Human Subjects Office will not relieve you of your responsibility to ensure compliance with Federal Regulations regarding annual review [as applicable, 21 C.F.R. § 56.109(f) and 45 C.F.R. § 46.109(e)].

You should retain a copy of this letter and all associated approved study documents for your records. Please refer to the assigned study number and exact study title in future correspondence with our office. Additional information is available on our website at at http://researchcompliance.iu.edu/hso/.

If your source of funding changes, you must submit an amendment to update your study documents immediately via an amendment.

If you have any questions or require further information, please contact the Human Subjects Office via email at irb@iu.edu or via phone at (317)274-8289.

Indiana University School of Dentistry

Graduate Student Research Proposal Abstract Form

(For review by IUSD IRB Committee)

Project Title: A visual comparison of oral plaque removal in the orthodontic patient utilizing an oral rinse with and without proteolytic enzyme prior to dental brushing and subsequent pulsating water jet cleansing

Name	Signature*
Principal Investigator: Kelton T. Stewart, DDS MS	
Co-Investigator: Jennifer Rose, DDS	
Co-Investigator: Frank Lippert, MSc, PhD	
Co-Investigator: Ahmed Ghoneima, BDS, MSc, PhD, MSD	
Research Committee: Lisa Maxwell, LDH, BS, MSM	
Biostatistician: George Eckert, MS	
Department/Program: Department of Orthodontics and Oral Facial Genetics	
Cost of Project:	$3500
Dates of Project:	April 2018-May 2018
Use of Human Subjects:	Yes
Use of Animals:	No
Use of Biohazards or rDNA:	No
Reviewed by Sue Kelly (IRB):	
Reviewed by Sue Kelly (Good Clinical Practice):	

*Signature indicates that the proposal has been read and approved.

Enzyme Research Protocol

Study Number:

Title: A Visual Comparison of Oral Plaque Removal in the Orthodontic Patient Utilizing an Oral Rinse with and without Proteolytic Enzyme Prior to Dental Brushing and Subsequent Pulsating Waterjet Cleansing

Phase: Not Applicable

Funding: WaterPik, Inc. (Providing free WaterPik devices only)
1730 East Prospect Road.
Fort Collins, Colorado 80553

Principal Investigator: Kelton T. Stewart, DDS MS
Department of Orthodontics
Indiana University School of Dentistry
1121 West Michigan St.
Indianapolis, Indiana 46202
Phone: (317) 278-1087
Fax: (317) 278-1438
E-mail: keltstew@iu.edu

Co-Investigator: Jennifer Rose, DDS
Department of Orthodontics
Indiana University School of Dentistry
1121 West Michigan St.
Indianapolis, IN 46202
Phone: (785) 760-6244
E-mail: jschaetz@iupui.edu

Co-Investigator: Frank Lippert, MSc, PhD
Oral Health Research Institute
415 Lansing St.
Indianapolis, IN 46202
Phone: (317) 274-3983
Fax: (317) 274-5425
E-mail: flippert@iupui.edu

Co-Investigator: Ahmed Ghoneima, BDS, MSc, PhD, MSD
Director, Orthodontic Clinic
Director, MSD Program
Department of Orthodontics
Indiana University School of Dentistry
1121 West Michigan Street, DS 249

Indianapolis, Indiana 46202
Phone: (317) 278-1653
Fax: (317) 278-1438
E-mail: aghoneim@iu.edu

Committee Member:

Lisa Maxwell, LDH, BS, MSM
Dental Hygiene Program Director
Indiana University School of Dentistry
1121 West Michigan Street, S408
Indianapolis, Indiana, 46202
Phone: (317) 274-2611
E-mail: lmax@iu.edu

Statistician:

George Eckert, MAS
Department of Biostatistics
Indiana University School of Medicine
410 W. 10th St.
Indianapolis, IN 46202
Phone: (317) 274-2884
Fax: (317) 274-2678
E-mail: geckert@iu.edu

IRB Advisor:

Sue Kelly
Director, Clinical Research Core
Indiana University Oral Health Research Institute
415 Lansing Street
Indianapolis, IN 46202
Phone: (317) 274-3954
E-mail: sakelly@iu.edu
Fax: (317) 274-5425

IRB:

IU Institutional Review Board
Human Subjects Office
Office of Research Administration, Room 3315
980 Indiana Ave.
Indianapolis, IN 46202
Phone: (317) 274-8289
Fax: (317) 274-5932
E-mail: resrisk@iupui.edu
FWA #: 00003544, exp IRB# Indiana University-
Purdue University at Indianapolis (IUPUI):

Version: April 5th, 2018

STATEMENT OF COMPLIANCE

Refer to:
http://www.hhs.gov/ohrp/humansubjects/guidance/45cfr46.htm#46
http://www.fda.gov/cder/guidance/959fnl.pdf
http:// grants.nih.gov/grants/guide/notice-files/NOT-OD-01-061.html
http://www.cancer.gov/clinicaltrials/learning/page3

The study will be carried out in accordance with good clinical practice (GCP) as required by the following:

United States (US) Code of Federal Regulations (CFR) applicable to clinical studies (45 CFR Part 46; 21 CFR Part 50, 21 CFR Part 56, and 21 CFR Part 312)

ICH E6; 62 Federal Register 25691 (1997)

Department of Orthodontics, Research Review Committee

All key personnel (all individuals responsible for the design and conduct of this study) have completed Human Subjects Protection Training.

SIGNATURE PAGE

The signature below constitutes the approval of this protocol and attachments, and provides the necessary assurances that this trial will be conducted according to all stipulations of the protocol, including all statements regarding confidentiality, and in accordance to local legal and regulatory requirements and applicable US federal regulations and IHC guidelines.

Signed: _____Date: _____
Name: Kelton T. Stewart, DDS MS
Title: Principal Investigator

Signed: _____Date: _____
Name: Jennifer Rose, DDS
Title: Student-investigator

Signed: _____Date: _____
Name: Frank Lippert, MSc, PhD
Title: Co-investigator

Signed: _____Date: _____
Name: Ahmed Ghoneima, BDS, MSc, PhD, MSD
Title: Co-investigator

Table of Contents

1. STUDY OBJECTIVE AND RATIONALE

1.1 Objective

The objective of this study is to determine if residual dental plaque following brushing can be mitigated by pre-rinse with broad spectrum oral proteolytic enzyme rinse and subsequent oral pulsating waterjet appliance, enhancing the oral hygiene of patients receiving orthodontic treatment.

1.2. RATIONALE

Since the advent of modern orthodontic treatment, maintaining optimal oral hygiene has been troublesome for both the patient and the clinician. Fixed orthodontic appliances in the oral cavity are a physical barrier which can interfere with optimal oral hygiene. The tooth surface beneath the wire and adjacent to the orthodontic bracket are challenging areas for the patient to properly clean. The placement of this hardware creates obstructions and obstacles to the abrasive function of the toothbrush bristles. Poor removal of plaque adjacent to the brackets often leaves a visible halo or shadow effect on the tooth surface when the orthodontic hardware is removed. This discoloration halo (white spot lesion) is due to acidic demineralization of the tooth surface beneath the adherent dental plaque. The tooth surface beneath the bracket is protected from plaque development and resultant acidic leaching of dental minerals. Upon bracket removal, the discrepancy in tooth mineralization between these adjacent areas is now exposed creating the unaesthetic white spots. Unfortunately, many orthodontic patients to some degree, develop these unaesthetic white spots on their teeth.

The purpose of this research study is to determine if utilizing a proteolytic oral pre-rinse can hydrolyze the glycoprotein bonds which adheres the dental plaque to the dental pellicle (tooth surface in vivo). It is proposed here that the proteolytic enzyme may loosen the bond enough to allow for dislodgement by standard brushing, and further reduction in oral plaque with subsequent water jet propulsion.

The question to be answered is thus: in an adolescent patient with orthodontic appliances and poor oral hygiene, can the water jet more easily reach those areas protected from the bristles of the toothbrush (created by the orthodontic hardware) and dislodge the enzymatically loosened plaque? If so, an enzyme pre-rinse may have a place in orthodontic hygiene procedures.

The findings of this study may lead to future research studies investigating whether the addition of proteolytic enzymes can also enhance the overall oral hygiene in the general population. Possibly removing plaque between teeth and other areas where brushing alone can't reach.

It is well known that upon initiation of fixed orthodontic appliance therapy there is a significant increase in dental plaque accumulation, in which patient education and oral hygiene instruction alone has proven insufficient to overcome(1). Poor oral hygiene during orthodontic treatment results in a high prevalence of white spot lesions on anterior tooth surfaces surrounding brackets. Martignon et al showed 96% of patients had one or more white spot lesions following orthodontic treatment (2).

The problem appears to be that the orthodontic wire and bracket impede the toothbrush bristles from close contact with an otherwise naturally smooth tooth surface. This hardware interferes with the mechanical contact and abrasive function of the toothbrush bristles to dislodge adherent dental plaque from the tooth surface. Residual plaque following brushing in orthodontic patients has been found along the gum line, around brackets and beneath wires(3).

The orthodontic wires make flossing an arduous task, which is why many patients give up and never adequately remove dental plaque from orthodontic hardware and dental contact points. Numerous studies have correlated fixed orthodontic treatment with less than optimal oral hygiene (4). Orthodontic induced gingivitis(5) (7) typically develops within the first few months of fixed appliance placement(6). Following the placement of the orthodontic fixed appliance, the resultant change in oral hygiene creates an environment favorable to *Streptococcus mutans*(8) and an increase in gram negative bacteria which are associated with periodontal disease(9). Improved oral hygiene would likely prevent many adverse sequelae commonly associated with orthodontic treatment (2).

Previous studies have used pre-rinsing agents as an oral hygiene adjunct and demonstrated mixed results. Boyd found Hydrogen peroxide-sodium fluoride rinse is more effective than sodium fluoride rinse alone in preventing gingivitis and decalcification in adolescent orthodontic patients(10). Miranda, conducted a trial using water, Listerine, or Plax, as pre-rinse agents and concluded that pre-rinsing in children demonstrated no difference in plaque removal (11). However, Tufekci showed the addition of Listerine rinse to an oral hygiene protocol significantly reduced plaque (more than just brushing and flossing alone in orthodontic patients). He concluded that the addition of Listerine was helpful for fixed orthodontic patients to reduce their risk of white spots development and gingivitis (12). Pontier's study concluded there was no difference between pre-rinse (Plax TM versus placebo) for plaque removal in orthodontic patients(13).

Water irrigation (pulsating water jet therapy), another adjunct to oral hygiene, has demonstrated a significant difference in biofilm (14) and plaque removal - especially in difficult to reach dental plaque retention areas, such as around the orthodontic brackets and wires (6). The plaque removal was also increased utilizing a specialized orthodontic water jet tip as compared to a standard water jet tip (15).

Surprisingly little has been investigated concerning proteolytic hydrolysis (to loosen the dental biofilm) influence on oral hygiene (16). No published studies could be located (PubMed) that involve use of proteases as a plaque removal aid in the orthodontic patient. Literature search revealed a study of Krillase (enzyme obtained from ocean krill) included in a chewing gum to reduce gingivitis and plaque acumination (17). Published articles utilizing the plant enzyme papain in orthodontics include: orthodontic bonding and sheer bond strength study (18) and the effect of different concentrations of papain gel have on orthodontic bracket adhesion study (19).

Papain and Bromelain are naturally occurring serine proteases. Papain is derived from the latex from green papaya fruit (*Carica papaya*) while bromelain is obtained from pineapple stalks (*Ananas comosus*). Because of their innate proteolytic activity, papain and bromelain have been used extensively in the food and medical industries. Both enzymes are included on the FDA's "Generally Recognized as Safe" (GRAS) list. Both bromelain and papain are used commercially for tenderizing meat and producing protein hydrolysates (20). These proteolytic enzymes have a broad range of substrate specificity, which easily and efficiently hydrolyze

most soluble proteins, yielding peptides and amino acids byproducts.

Other uses of these enzymes are in toothpaste (papain/bromelain combination as the active ingredient) for extrinsic enamel stain removal (21, 22)(23). These enzymes have also been used for wound debridement for of damaged collagen and necrotic tissue in humans (24). Finally, health food enthusiasts claim dietary supplementation with papain and bromelain may be useful in alleviating joint pain and inflammation and supplementing natural digestive proteases.

1.3. Potential Risks and Benefits

1.3.1. Potential Risks

Product Risks

Irritation of the oral cavity can occur, (also known as stomatitis or angular chelitis), and has occurred among those individuals who eat extraordinary amounts of fresh pineapple. The use of an active protease in an oral rinse may cause stomatitis or angular chelitis and possibly irritate an undetected oral ulcer and cause the subject discomfort.

Adverse reactions

Hypersensitivity/allergic reactions have been reported in individuals who have supplemented their diet with bromelain and are allergic to pineapple. Nausea, vomiting, and diarrhea were reported in such instances following ingestion.

Contraindications

Use of bromelain is contraindicated in those who are hypersensitive or allergic to pineapple or proteases. Gastrointestinal or oral ulcers, use of anticoagulant/blood thinner medication, antibiotics or anticipating surgery would also be contraindications to ingestion of active proteases.

Precautions

Bromelain has a long history of being used in the food industry and no serious side effects or deaths have been reported. Nevertheless, certain precautions will be taken in using this natural protease.

Rinses will be expectorated. Only a negligible amount of enzyme may be ingested unless the test subject inadvertently swallows the solution. Even in this scenario, this should not be a problem since quantities in greater amounts are frequently ingested by individuals wishing to supplement their natural pancreatic digestive capabilities.

Interactions

Since bromelain is an active serine protease, it is susceptible to inactivation or denaturation by excessive heat, chemicals or ions. Therefore, the oral rinse test solutions will not have alcohol or fluorine compounded within the solution tested.

Over dosage

Bromelain has been shown to be safe when ingested in less than 130,000 FCC papain units per day (approximately 1.5 g of enzyme preparation). Symptoms of over dosage using bromelain as an oral rinse would include development of oral ulcers, stomatitis or angular chelitis. This would produce mild discomfort for a few days while the oral mucosa regenerates.

Additional Risks

Additional potential risk to the subjects include cross contamination. This risk will be managed by the use of experienced IU dental personnel proctoring the test subjects, whom will follow strict infection control guidelines outlined by the IU School of Dentistry. This includes handwashing between subjects, use of single use nitrile gloves, and facemasks (for researchers) during the intraoral exam. Vials and cheek retractors will be single use.

Loss of confidentiality of private information collected is another risk. To counter this risk, all data collected on paper will be stored in a locked area and limited to study personnel. Electronic data will be stored in an encrypted, password protected computer file with access limited to study personnel. All records will be identified by study number only. A master list will be maintained which will match the subject to their study number and this list will be stored in a secured area.

1.4. Adequacy of Protection against Risks

To limit the potential risks of ingestion of the active ingredients, persons with the following conditions will be excluded from participation in the study:

1. gastrointestinal or oral ulcer
2. taking an anticoagulant/blood thinner medication or antibiotics
3. anticipating surgery in the next six months
4. allergic to proteolytic enzymes, pineapples or disclosing agent
5. pregnant, breastfeeding or intending to become pregnant in the next six months

1.5. Known Potential Benefits

There is no known benefit for the subject.

2. STUDY DESIGN

This will be a single site, cross-over, double-blind study with up to 55 subjects enrolled in the study. Since each person varies with technique and diligence in brushing their teeth, each subject will serve as their own control in this cross-over study. There will be at least a week time interval between the two arms of the study (length of wash out phase: one week). The study will be completed over the course of two weeks. Each step in the protocol will be timed to reduce inter- and intra-subject variability. Toothbrushes used in the study will be uniform, a new tooth brush will be used at each arm of the study. All oral hygiene aids will be of the same brand to eliminate variability (eg: all toothbrushes will be of the same brand, all disclosing tablets of the same brand, etc). For standardization and optimal photography, the same type of cheek retractor and digital camera will be used for all subjects.

Hypothesis

Null: There is no statistically significant difference in visual plaque scores with or without use of a proteolytic enzyme rinse aid.

Alternative: There is a statistically significant difference in visual plaque scores with use of proteolytic enzyme rinse aid.

Product Description

This study will use bromelain as the proteolytic enzyme in the oral rinse. Bromelain is an enzyme naturally found in high concentration in the stem of pineapples. This proteolytic, protein-digesting enzyme breaks down (hydrolyzes) proteins by reducing large complex proteins into smaller peptides or individual amino acids (25). For this reason, bromelain is commonly used as a digestive aid, (in pill form) or (in powder form) as a meat tenderizer.

2.1. Study Schedule

In both arms of the study, the steps will be exactly the same to isolate the effect of the enzyme. Data (digital photograph) will be obtained after each oral hygiene step to prevent confounding variables.

First Visit

a. Describe study and obtain informed consent, if not previously consented by phone(see attachment).
b. An oral soft tissue exam will be completed (see section 7.4).
c. Randomize subject and select a subject kit (test box) Patient writes name on outside of box and on WaterPik box. The patient name on outside of box allows easy retrieval for second visit.
d. Stain oral plaque with two-tone plaque disclosing tablet (individually packaged disclosing tab). Instructions for test subject using oral disclosing agent: "chew tablet completely, then swish around in your mouth and between your teeth. I will time you for 30 seconds and tell you when to spit it out in the sink." Rinse with 15ml of tap water to remove unbound stain.
e. First photograph (base line plaque before oral hygiene procedures) obtained with unique identifying number (see 9.4).
f. Subject chooses one of the test vials from their box and the serial number on the vial is recorded (placebo rinse or enzymatic rinse). (The second vial will be used on the second testing visit.)
g. The student investigator will reconstitute oral rinse powder in vial with 15 mL-warm (50°C) pineapple juice from coffeepot on hotplate. Stir into solution with disposable swizzle stick.
h. Hand the vial with the dissolved oral rinse solution to the subject. The subject will then place the entire contents at once into their mouth and swish it between their teeth and side to side for two minutes (timed). At the end of two minutes the subject expectorates contents of mouth into sink.
i. Second frontal photograph obtained (post-rinse). (This photo will include a different random identifying number unique for subject, stage and visit).
j. The subject will then be asked to brush their teeth with a new toothbrush (individually packaged and pre-pasted from manufacturer). The only instructions in regards to brushing: "brush your teeth as you normally do." Subject brushing time will be standardized (two minutes) and a digital alarm clock will keep time. The subject will expectorate contents of mouth at the end of two minutes.
k. Third frontal photograph again will be obtained including another identifying number specific to this stage of the experiment and visit.
l. The subject will then be asked to use a water jet cleansing device (WaterPik). This will also

be timed (two minutes). The instructions given to the subject: "Use this Waterpik to clean around the brackets and under the wires as best you can". All Waterpik devices will be set at the same medium pulsating pressure.

m. Fourth frontal photograph for this visit obtained (including another random serial identifying number unique for subject, stage and visit).
n. Subject receives $25 Visa gift card and dismissed.
o. Subject (or guardian) is phoned later that day & questioned if they have any concerns or side effects. Subject & Guardian are reminded of phone number contact of student researcher.

Second Visit (1week later)

a. Each subject will repeat the procedure as described above on the subsequent visit (a-n) with the second (vial) oral rinse treatment (placebo or enzyme).

3. STUDY POPULATION

3.1. Source and Number of Subjects

55 adolescent and young adult subjects (10-25 years, male or female). Subjects will be in active fixed (edgewise) orthodontic appliances at the Indiana University School Dentistry.

3.2. Subject-Selection Criteria

Inclusion Criteria
Individuals may qualify to participate in the study if they meet all the following requirements:

- Subject is willing to consent
- male or female
- age range from 10-25 years
- willing to follow study instructions
- in active orthodontic treatment with fixed orthodontic appliances
- patient at Indiana University School of Dentistry Orthodontic Clinic

Exclusion Criteria
Individuals will be excluded from participation if they possess any of the following conditions:

- pregnancy, breast feeding or intending to become pregnant in the next six month period
- latex allergy
- not willing/able to follow study instructions
- pineapple allergy
- proteolytic enzymes allergy
- food dye allergies (to assure no issues with using disclosing solution)
- smoker
- has a gastrointestinal or oral ulcer
- is taking an anticoagulant/blood thinner medication or antibiotics
- is anticipating surgery in the next six months
- person with dental training (dental students, hygiene students, faculty, & assistants)

3.3 Subject Recruitment

Potential subjects will be recruited from patients at the Indiana University School of Dentistry Orthodontic Department. Recruitment methods will include the following:

1) An IRB approved flyer will be hung in the waiting room and various locations within the clinic.
2) The student investigator will call patients who are her patients in the clinic to determine interest.
3) All residents and faculty will assist in distributing an IRB approved flyer that contains a section with contact information for the subject to complete if they are interested in learning more about the study. Potential subjects will be able to place completed interest forms with their contact phone number in a secured drop box in the clinic for the student investigator to access. The student investigator will then call the potential subjects to discuss the study.

In all cases, the student investigator will use an IRB approved telephone script to review the study with the potential subjects. If the potential subject is interested in participating, and if time allows, the consent and assent will be sent to the potential subject by mail so they will have the opportunity to review the information before their appointment. Consenting and assenting, as applicable, will occur in the clinic prior to any study procedures being performed. Consenting may also occur by phone, using the consent documents sent by mail. Test subjects will attend two testing visits approximately one week apart. These visits will be outside from their normal orthodontic visits.

Subjects will be compensated for their time in the study by gift of the testing Waterpik appliance they used and $50 ($25 per visit) for participating in the study. Payment will be in the form of Visa gift cards.

4. TEST TREATMENTS

4.1. Administration of Study Investigational Product

The components of each treatment will be formulated prior to treatment and placed in a plastic vial. Each of the vials for the oral rinse treatments will be labeled with a unique set of numbers for subject and stage of treatment (only decoded by use of master list).

4.2. Blinding/Unblinding Procedures

To eliminate potential human bias, photos will be graded independently by one examiner from Indiana University School of Dentistry with dental clinical experience. The examiner will be blinded with regard to the stage and visit of the oral hygiene procedures. The identification on the photos will not allow the examiner to know which photos came from the same subject, which series they are evaluating (baseline staining, post-rinse, post-brush, post-Waterpik) and which treatment.

Each photo will be independently reviewed and scored assessing the degree of plaque staining from one to five. An example photograph of each degree (1-5) will be given to the examiner for standardization.

The researchers and subjects will be blinded to knowing which vial has the proteolytic enzyme treatment. The subsequent visit the subject will receive the second vial. The study records will be maintained by this committee member (DR. KS).

Each photograph will be labeled by only a serial number and can only identified through use of a master sheet. Each subject will have eight randomly assigned serial numbers corresponding to each testing step and visit (two different study visits with four photographs each).

4.3 Photo Labeling

A photographic sequence identifier (A-D) will be added to the patient's random subject ID to properly log and sequence the obtained photographs. The following codes will be utilized in the study:
A: After use of the disclosing tablet
B: After 2 minute rinse with provided solution
C: After brushing
D: After the use of the WaterPik

4.4. Treatment Compliance

It is expected most subjects enrolling in the study will complete both treatment visits. Since the treatments are to be administered to the subject and supervised, compliance with the protocol is expected.

4.5. Randomization Procedures

Subjects will be stratified randomly by sex and age groups. Subjects will be allocated random sets of serial numbers kept on a master list which will be retained in a secured location. The faculty member grading the photographs will be given the 440 photographs completely in random order, randomized by the primary investigator.

5. STUDY METHODOLOGY

5.1. Preparation of Investigational Products

The dry components of each rinse will be formulated prior to study and placed in a plastic vial with lid. The statistician will generate a second randomization list to identify which vial (A or B) the patient will receive at the first and second visits, respectively.

Prior to beginning the study, the dry powdered components for each treatment rinse will be weighed and placed into clean 20 mL vials, (for both the enzymatic and placebo rinse) and sealed with plastic caps. This will prevent contamination or spillage. All vials will be prepared at one time in a clean room prior to the study.

Selected committee members will measure out one gram of enzyme and one gram of placebo (powdered sugar) for each subject. Both the placebo and the proteolytic rinse compounds will be weighed, formulated and placed into darkened 20 mL vials with a plastic top. Each vial will then be assigned the appropriate code (Product A or Product B) by the co-investigator K.S. Only this investigator (K.S.) will know the identity of the vial contents and will share this information with the statistician at the time of statistical analysis. The formulated compounds placed in the clean vials will be stored in a locked secured location.

<u>Manufacture of Bromelain Rinse:</u>

The bromelain enzyme will be obtained (donated) in bulk powder form from Ultra Bio-logics Inc. (Chateauguay, Quebec, Canada). The Ultra Bio-logic bulk bromelain concentrated supplement is standardized (by manufacturer) to 2400 Gelatin Digesting Units (GDU)/gram. The strength of an enzyme is quantified in GDU/gram units. GDU/gram tells us how much protein a gram of bromelain can digest. The proteolytic rinse will contain one gram of bromelain brought into solution with 15ml of pineapple juice. One gram of bromelain is within the recommended daily dosage for oral consumption by the manufacturer.

Bromelain is stable at room temperature for more than a year in its dry form. It is active when reconstituted with warm water. (In this study- warm pineapple juice will be substituted for warm water). Since bromelain is active in the liquid form, the rinse will be constituted with canned pineapple juice just prior to administration while ensuring complete dissolution. Bromelain's enzymatic activity is destroyed by the pasteurization process during canning. Thus, the canned pineapple juice has the correct pH for optimal activity but does not contribute any enzymatic activity to the test solution.

Bromelain's optimal activity is at 55°C. This is approximately the temperature of very warm coffee. To achieve optimal results a temperature controlled hot plate will be used to keep a pot of warm pineapple juice at 50°C available to mix with the powdered mixture just prior to administration.

Manufacture of Placebo Rinse:

The placebo rinse will consist of powdered sugar and pineapple juice. Powdered sugar has the same color and consistency as the bromelain powder. This placebo will not be discernible once weighed and placed in sealed vials. Canned pineapple juice contains no active bromelain, which is destroyed in the pasteurization process.

The components of the two study rinses are as follows:

TREATMENT A: Placebo	TREATMENT B: Proteolytic Rinse
Pineapple juice (15 mL)	Pineapple juice (15 mL)
Powdered sugar (1.0 gm)	Bromelain powder (1.0 g)

5.2. Clinical Procedures

All study materials needed for each test subject will be contained in a new cardboard box. Each box will contain:

- Vial of enzyme powder with attached set of assigned random numbers
- Vial of placebo powder with attached set of assigned random numbers
- 10 disposable cheek retractors
- Two disclosing tablets for staining
- Waterpik device
- Two toothbrushes (pre-pasted and individually sealed by manufacturer)

After explaining the experiment in obtaining consent (either by phone or in person), the subject will be randomized and given their assigned study box. The subject will then put their name on the outside of the box and on the box of the Waterpik. The name on the outside of the box will allow for retrieval for the second visit. The student researcher will then record the first serial number with the name of the test subject. This will be entered on the master list. The master list will then have been

pre-prepared and grouped into 55 patient sets. Each set of random numbers for each patient will be divided and assigned with four numbers attached to each test vial. At the completion of the first visit, the cardboard box containing the Waterpik and the remaining materials will be sealed with adhesive tape and secured in a locked room. When the patient returns for their next testing visit, that subjects' box will be retrieved and the second vial labeled with the subject's second set of numbers will then be used. The test procedure will be repeated as with the first visit as described in 2.1.

5.3. Informed Consent Process

Informed consent will be obtained from subjects and two parents (if the subject is a minor <18 yrs and if 2nd parent is reasonably available within the CFR regulations) prior to participation in study either in person or by phone (see 10.2). Assent will be obtained from all children under age 18. For children 14 to 17, the adult consent will be used as the assent form.

5.4. Oral Photography Process

Standardization of photographs: all photographs will be taken from the same angle (frontal), to include only the teeth and lips of the subject, with a single use, hands-free cheek retractor. Subject will be partially open so that the lower dentition is fully visible for photography. Each photograph will be at the same magnification (see 10.4). The adhesive label with the corresponding serial number will be placed on the subject's chin and will be included in the photograph.

6. EFFICACY MEASUREMENTS AND EVALUATIONS

6.1 Training & Calibration of Examiner:

One examiner from the Department of Periodontics and Allied Dental Programs or Cariology, Operative Dentistry and Dental Public Health, will be recruited to assess the plaque index of the digital photographs. An example of five oral photographs with varying degrees of stained adherent dental plaque will be graded according to the Orthodontic Plaque Index and used as a reference in grading the study photographs. The examiner will independently grade the residual plaque in each photograph.

The examiner will first be calibrated by scoring 10 randomly selected images and re-score the same images after a one week washout period. Once reliability testing of the examiner is at 0.8 or higher as measured by the intra-class correlation coefficient, the examiner will proceed to judge each photograph of the study.

6.2. Dental Plaque Evaluation and Data Management

Teeth will be disclosed and the facial tooth surfaces will be graded for visual plaque.
Incisors (#s: 7-10 and 23-26) will be visually evaluated on digital photographs and assigned a score (0-4) according to the orthodontic plaque index (see figure 1 below). Examiner will be given a visual scale of comparison, which corresponds with the Orthodontic Plaque Index (OPI) for diagnosing dental plaque around orthodontic brackets. Each tooth will each receive an individual score, in addition all tooth scores will be added together to calculate a composite score for upper incisor and lower incisor segments. All recorded plaque scores will be recorded in an excel document on a password protected laptop computer by the grader.

To eliminate potential human bias, a single examiner from the Indiana University School of Dentistry with clinical dental experience, will judge each photograph. This examiner will be

blinded with regard to the stage of the oral hygiene procedure as described above.

Data/digital photographs will be viewed on the same computer and will be backed up daily onto a secure box.iu.edu health account, which will only be accessible to researchers and by two step authentications and passphrase. Research laptop will remain in secure location and locked whenever not in use.

Plaque scoring:

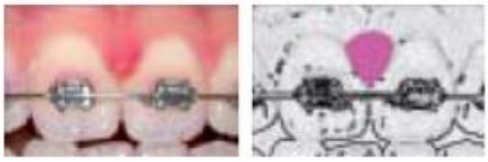
OPI score	Example	Description
0		Brackets are plaque-free
1		Isolated plaque islands on one tooth surface at the bracket base
2		Plaque on two tooth surfaces at the bracket base
3		Plaque on three tooth surfaces at the bracket base
4		Plaque on all tooth surfaces at the bracket base
		and/or gingival inflammation

Figure 1: Orthodontic Plaque index scoring system(Beberhold).

6.3. Retention of Photographs

Digital photographs and any study material will be maintained for seven years following the completion of this study, as per Indiana law. Paper records will be stored in locked cabinets that only study personnel can access. Electronic records will be stored in encrypted, password protected files only accessible by study personnel.

7. ASSESSMENT OF SAFETY

7.1. Study Safety Management

The PI will be notified immediately if a serious adverse event occurs. An adverse event will be considered any unfavorable and unintended sign, symptom or disease associated with the use of the bromelain (investigational product) or the conduct of the study.

All adverse events will be reported to the IRB during annual review.

All serious adverse events will be reported immediately to the IRB. Serious adverse events include: any event resulting in death, is life-threatening, requires hospitalization, results in disability/permanent damage, congenital anomaly/birth defect, or requires intervention to prevent permanent impairment or damage, and/or other medical events(31).

7.2. Medical/Dental Monitor

The principal investigator and student investigator will be responsible for overseeing the conduct of the study.

7.3. Specification of Safety Parameters

The temperature of the pineapple juice (to reconstitute the solutions) will be monitored to make sure it is 50°C plus or -1°C. The formulated compounds placed in the clean vials will be stored in a locked secured location.

7.4. Methods for Assessing Safety Parameters

Each subject will be given the student investigator's phone number to call if they should experience any discomfort or unusual symptoms. On the second visit each subject will be questioned as to whether they experienced any discomfort or symptoms from the treatment at the first visit and an oral soft tissue exam will be completed.

7.5. Safety Oversight Plan

All subjects will receive a follow- up phone call the night of the study and reminded of the contact phone number of the student investigator in case of emergency or adverse side-effects. Subject will be instructed to make contact, should they experience any discomfort or unusual symptoms. This is part of the study's safety plan to assure the subjects are not reacting negatively to the product.

On the second visit each subject will be questioned as to whether they experienced any discomfort or symptoms from the treatment (symptoms of over dosage using bromelain as an oral rinse may include development of oral ulcers, stomatitis or angular chelitis) at the first visit & an oral soft tissue exam will be completed.

If a subject experiences unusual side effects or appears to be experiencing an allergic reaction the subject will immediately be excluded from the study and will seek medical care if indicated.

8. DATA ANALYSIS

8.1. Sample Size Determination

55 subjects will be enrolled to account for dropout. Standard deviation of the average plaque scores is assumed to be 1, with a correlation of 0.5 between the two study arms. With a minimum completed

test sample size of 45 subjects, the study will have 90% power to detect a difference of 0.5 between the average plaque scores with and without enzyme after waterjet, assuming a two-sided paired t-tests conducted at a two-sided 5% significance level.

8.2. Statistical Analysis

Intra-examiner repeatability of the individual surface scores will be evaluated using weighted kappa statistics and repeatability of the subject-level index scores will be evaluated using intra-class correlation coefficients (ICCs) and Bland-Altman plots. Subject-level plaque scores will be summarized by treatment (with or without enzyme) and stage (baseline, post-rinse, post-brushing, post-waterjet). The effects of the treatment will be evaluated using a repeated measures ANOVA suitable for a crossover study. The ANOVA will include fixed effects for treatment, stage, and their interaction, will treat stage as a repeated factor within subject for each study arm, and will include a factor for study arm. Age and sex groups will be included as covariates due to the stratified randomization. The ANOVA will also include an effect for treatment sequence to evaluate the model for a carry-over effect. If the treatment sequence effect is significant, the full analysis may need to be interpreted cautiously or the analysis may need to be limited to using only the results from the first study arm. A 5% significance level will be used for all tests. Statistical analyses will be performed using SAS version 9.4 (SAS Institute, Inc., Cary, NC).

9. OBLIGATION OF THE INVESTIGATOR

9.1 Institutional Review

The proposal and protocol will be reviewed within the School of Dentistry. After it is approved by the School of Dentistry the proposal will be submitted to the Institutional Review Board for their approval.

9.2 Subject Assent

Informed assent will be obtained from subjects (who are minors) prior to participation in study.

9.3 Subject Consent

Informed consent will be obtained from subjects (and both parents of minors unless, per CFR regulations, only one parent signature is needed) prior to participation in study.

9.4 Data Collection and Measurements

Digital photography will make up the data for this study. Digital photography will be recorded at each step, for each subject-for evaluation of facial surfaces of anterior incisors (upper and lower). An intraoral dental plaque disclosing agent will be used to stain the oral plaque present, which will be documented via digital photography.

This study involves two separate visits. At each visit there will be a total of four photographs obtained. Each time the subject's teeth are photographed it will involve a frontal photograph to include only the facial surfaces of the teeth of each subject. Lingual surfaces of teeth will not be taken into account as the focus of this study relates to oral hygiene procedures around fixed appliances- all fixed appliances in this study will be on facial surfaces.

Each subject will have eight unique serial numbers assigned to them to differentiate the four different photograph at the two separate visits. A hands free cheek retractor will be utilized for all photographs. For consistency, fixed appliances including orthodontic wires will remain in place throughout the study.

The same type of camera (Canon Rebel EOS with 100mm macro lens) and camera settings (f-stop: 32, shutter speed: 1/60, ISO: 200) will be used for each subject at every data collection. Photographs will be taken at the same magnification and focus. The student investigator will review all photos immediately following to ensure that they are of diagnostic quality.

Diagnostic quality of data (digital photos of dentition) will be defined as non-blurry digital photos of dentition of upper and lower incisors with orthodontic fixed appliances in place. The dentition visualized will be the same in each subject and for both study visits. Facial surfaces of upper and lower incisors will be clearly and completely visualized in the digital photos. No lips will obstruct the tooth in the photo. Orthodontic appliance will remain on the teeth which are photographed and plaque surrounding the bracket and wire will be graded according to visual plaque index.

Data/photo collection will be standardized so that all photos are taken at the same step in the hygiene process/step of protocol (in this way, a photo is taken after each oral hygiene procedure to evaluate any change in amount of dental plaque) (See Flow Chart in Appendix, page 22).

1) Baseline plaque photograph following chewing/spitting of disclosing tablet.

2) Post-treatment rinse (enzyme or placebo) photograph.

3) Post- brushing photograph.

4) Post-Waterpik photograph.

Each photograph will be independently reviewed using the same computer and scored assessing the degree of plaque according to the Orthodontic Plaque index method by a single examiner.

9.5 Adherence to Protocol

Strict adherence to the protocol is essential to eliminate variables which would invalidate the study design. Subjects unwilling or unable to follow the protocol will be eliminated from the study.

9.6 Records Retention

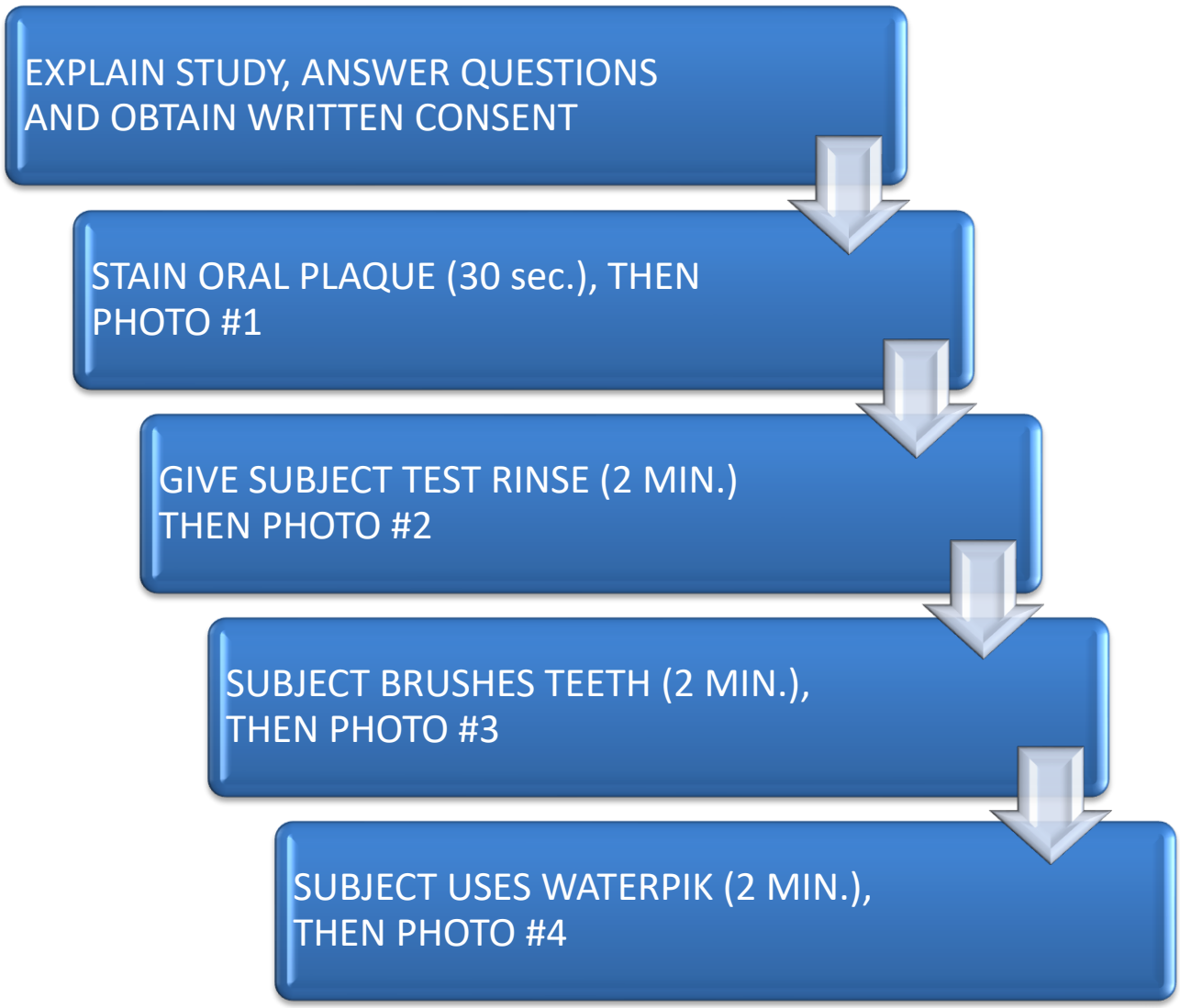
A digital copy of all the photographs and scoring will be retained by the principal investigator for no less than seven years, as per Indiana law.

9.7 Investigator's Final Report

The data and scoring from each subject will be compared with respect to each arm of the study. A report will be written with the results and statistical analysis of the study results. A paper will be written and submitted for publication with the summation of the findings.

APPENDIX

PROCEDURE FLOWCHART



Indiana University School of Dentistry
Student Research Proposal
Budget Worksheet

Name: Jennifer Rose, DDS

Title of Proposal: A visual comparison of oral plaque removal in the orthodontic subject utilizing an oral rinse with and without proteolytic enzyme prior to dental brushing and subsequent pulsating water jet cleansing

Anticipated Start Date: April 2018
$300 Requested from Research Committee
$3200 supplied "in kind" from Other Sources

Budget.	**Description:**	**Amount ($):**	**Source:**
Materials:	SIM card	$40	IU research committee Funds
	Toothbrushes ("Ready Brush" disposable, individually wrapped & pre-pasted)	$100	
	Disclosing tablets (Two tone chewable by Plaqsearch)	$30	
	Vials	$100	
	Recruiting flyers	$50	
	Pineapple juice	$10	
	Powdered Bromelain	$50	
	Poster fabrication	$50	Donated by IUSD
	Waterpik®s (55)	$1925	Donated by Waterpik
	Digital Camera: (Canon Rebel EOS) 100mm macro lens		Borrowed from IUSD Orthodontic Department
	Cheek retractors	$100	Will be purchased from Amazon & disposed of after one use
Panelist/Subject Pay:	$50 gift card	$2750	Grant from Delta Dental
Biostatistics:	Statistical analysis	$3200	IU research committee

12. **REFERENCES**……

1. Hagg U, Kaveewatcharanont P, Samaranayake YH, Samaranayake LP. The effect of fixed orthodontic appliances on the oral carriage of Candida species and Enterobacteriaceae. European journal of orthodontics. 2004;26(6):623-9.
2. Martignon S, Ekstrand KR, Lemos MI, Lozano MP, Higuera C. Plaque, caries level and oral hygiene habits in young patients receiving orthodontic treatment. Community dental health. 2010;27(3):133-8.
3. Klukowska M, Bader A, Erbe C, Bellamy P, White DJ, Anastasia MK, et al. Plaque levels of patients with fixed orthodontic appliances measured by digital plaque image analysis. American journal of orthodontics and dentofacial orthopedics : official publication of the American Association of Orthodontists, its constituent societies, and the American Board of Orthodontics. 2011;139(5):e463-70.
4. Wites M, Panuszka J, Dyras M. [Evaluation of oral and orthodontic appliance hygiene in orthodontically treated patients]. Przeglad lekarski. 2003;60 Suppl 6:126-8.
5. Ristic M, Vlahovic Svabic M, Sasic M, Zelic O. Clinical and microbiological effects of fixed orthodontic appliances on periodontal tissues in adolescents. Orthodontics & craniofacial research. 2007;10(4):187-95.
6. Attarzadeh F. Water irrigating devices for the orthodontic patient. International journal of orthodontics. 1990;28(1-2):17-22.
7. Zurfluh MA, van Waes HJ, Filippi A. The influence of fixed orthodontic appliances on halitosis. Schweizer Monatsschrift fur Zahnmedizin = Revue mensuelle suisse d'odonto-stomatologie = Rivista mensile svizzera di odontologia e stomatologia. 2013;123(12):1064-75.
8. Attin R, Thon C, Schlagenhauf U, Werner C, Wiegand A, Hannig C, et al. Recolonization of mutans steptococci on teeth with orthodontic appliances after antimicrobial therapy. European journal of orthodontics. 2005;27(5):489-93.
9. Rego RO, Oliveira CA, dos Santos-Pinto A, Jordan SF, Zambon JJ, Cirelli JA, et al. Clinical and microbiological studies of children and adolescents receiving orthodontic treatment. American journal of dentistry. 2010;23(6):317-23.
10. Boyd R. Two-Year Longitudinal Study of a Peroxide-Fluoride Rinse on the Calcification in Adolescent Orthodontic Patients. Journal of Clinical Dentistry 1992;3(3): 83-7.
11. Miranda Rda S, Marques RA, Dummel C, Soares FZ, Oliveira MD, Rocha Rde O. The influence of prebrushing mouthwashes on plaque removal in children. Pediatric dentistry. 2014;36(3):211-5.
12. Tufekci E, Casagrande ZA, Lindauer SJ, Fowler CE, Williams KT. Effectiveness of an essential oil mouthrinse in improving oral health in orthodontic patients. The Angle orthodontist. 2008;78(2):294-8.
13. Pontier JP, Pine C, Jackson DL, DiDonato AK, Close J, Moore PA. Efficacy of a prebrushing rinse for orthodontic patients. Clinical preventive dentistry. 1990;12(3):12-7.
14. Kato K, Tamura K, Nakagaki H. Quantitative evaluation of the oral biofilm-removing capacity of a dental water jet using an electron-probe microanalyzer. Archives of oral biology. 2012;57(1):30-5.
15. Gorur A, Lyle DM, Schaudinn C, Costerton JW. Biofilm removal with a dental water jet. Compendium of continuing education in dentistry (Jamesburg, NJ : 1995). 2009;30 Spec No 1:1-6.
16. Daep CA, Novak EA, Lamont RJ, Demuth DR. Selective substitution of amino acids limits proteolytic cleavage and improves the bioactivity of an anti-biofilm peptide that targets the periodontal pathogen, Porphyromonas gingivalis. Peptides. 2010;31(12):2173-8.
17. Hellgren K. Assessment of Krillase chewing gum for the reduction of gingivitis and dental plaque. The Journal of clinical dentistry. 2009;20(3):99-102.
18. Agarwal RM, Yeluri R, Singh C, Munshi AK. Enamel Deproteinization using Papacarie and 10% Papain Gel on Shear Bond Strength of Orthodontic Brackets Before and After Acid Etching. The Journal of clinical pediatric dentistry. 2015;39(4):348-57.
19. Pithon MM, Ferraz CS, Oliveira GD, Dos Santos AM. Effect of different concentrations of papain

gel on orthodontic bracket bonding. Progress in orthodontics. 2013;14:22.
20. : Enzyme Development Corporation; [Available from: http://www.enzymedevelopment.com/applications/tenderizer/.
21. Chakravarthy P, Acharya S. Efficacy of extrinsic stain removal by novel dentifrice containing papain and bromelain extracts. Journal of young pharmacists : JYP. 2012;4(4):245-9.
22. Kalyana P, Shashidhar A, Meghashyam B, Sreevidya KR, Sweta S. Stain removal efficacy of a novel dentifrice containing papain and Bromelain extracts--an in vitro study. International journal of dental hygiene. 2011;9(3):229-33.
23. Patil PA, Ankola AV, Hebbal MI, Patil AC. Comparison of effectiveness of abrasive and enzymatic action of whitening toothpastes in removal of extrinsic stains - a clinical trial. International journal of dental hygiene. 2015;13(1):25-9.
24. Goldman M, Kronman JH. A preliminary report on a chemomechanical means of removing caries. Journal of the American Dental Association (1939). 1976;93(6):1149-53.
25. Group DE. The Health Benefits of Bromelain: Global Healing Center; [Available from: https://www.globalhealingcenter.com/natural-health/bromelain/.
27. Keukenmeester RS SD, Rosema NAM, Van der Weijden GA. . Determination of a Comfortable Volume of Mouthwash for Rinsing. . International Journal of Dental Hygiene. 2012;10:169-74.
28. Al-Anezi SA, Harradine NW. Quantifying plaque during orthodontic treatment. The Angle orthodontist. 2012;82(4):748-53.
29. Thienpont V, Dermaut LR, Maele GV. Comparative study of 2 electric and 2 manual toothbrushes in patients with fixed orthodontic appliances. American Journal of Orthodontics and Dentofacial Orthopedics. 2001;120(4):353-60.
30. Clerehugh V, Williams P, Shaw WC, Worthington HV, Warren P. A practice-based randomised controlled trial of the efficacy of an electric and a manual toothbrush on gingival health in patients with fixed orthodontic appliances. Journal of Dentistry. 1998;26(8):633-9.
31. FDA. What is a Serious Adverse Event?
32. Arici S, Alkan A, Arici N. Comparison of different toothbrushing protocols in poor-toothbrushing orthodontic patients. European journal of orthodontics. 2007;29(5):488-92.

Beberhold, Katrin & Sachse-Kulp, Anika & Schwestka-Polly, Rainer & Hornecker, Else & Ziebolz, Dirk. (2012). The Orthodontic Plaque Index: An oral hygiene index for patients with multibracket appliances.. Orthodontics : the art and practice of dentofacial enhancement. 13. 94-9.