Effect of an active compound containing gum on dental plaque formation on a 4-day accumulation model

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1.0 Abbreviations

AE	Adverse Event
Am	Ante Meridiem
cc	Cubic Centimeter
CRF	Case Report Form
eCRF	Electronic Case Report Forms
FDA	Food and Drug Administration
GCP	Good Clinical Practice
HIPAA	Health Insurance Portability and Accountability Act
IRB	Institutional Review Board
IUSD	Indiana University School of Dentistry
Med Hx	Medical History
Mm	Millimeter
OH	Oral Hygiene
OHRI	Oral Health Research Institute
OHT	Oral Hard Tissue
OST	Oral Soft Tissue
PI	Principal Investigator
PLI	Plaque Index
Pm	Post Meridiem
QA	Quality Assurance
QC	Quality Control
SAE	Serious Adverse Event
SAP	Statistical Analysis Plan
SOP	Standard Operating Procedure
TMD	Temporo Mandibular Disorder
TMJ	Temporo Mandibular Joint
Tx 1	Treatment 1 (Experimental Regimen)
Tx 2	Treatment 2 (Positive Control Regimen)
Tx 3	Treatment 3 (Control Regimen)
UP	Unanticipated Problem

2.0 Background & Rationale

The oral cavity harvests many microbial species that have a protective function against pathogenic foreign microbes that can compromise oral health. Due to its nature, this is a dynamic, stratified environment that can quickly lose its balance. Oral biofilms can shift composition due to local or systemic challenges, becoming a contributory etiology to some of the most common global oral diseases, dental caries, and periodontal conditions.[1]

There is a general understanding that the oral biofilm of a healthy individual primarily contains bacterial species such as *Neisseria, Streptococcus, Actinomyces, Veillonella, and Granulicatella*. However, these species are also found in the dental pathologies previously mentioned. The pathogenesis of these microorganisms is exacerbated by the nutrients found in saliva, changes in microbial cooperations, and the host immune responses between others. If untreated, the result would be tooth loss, which can greatly affect a patient's quality of life. [2]

Controlling dental plaque formation and proliferation has been a significant goal for dental professionals. Several methods, including mechanical, chemical, and educational interventions, have been presented and have produced good results. However, these interventions have limitations, especially those related to mechanical plaque removal. The patient's dexterity during toothbrushing and interdental flossing procedures can play an important role in the patient's oral hygiene. [3-5] Alternative methods for plaque removal may benefit some risk populations, such as the elderly, patients with disabilities, and those without access to clean water. [6-8]

Chewing sugar-free gum can increase salivary flow, which will favor a more neutral dental plaque pH, facilitate the natural cleansing of sugars, and favor enamel remineralization.[9, 10] Also, adding other products to the gum can significantly impact the accumulation of dental plaque and decrease gingivitis in patients. [11, 12]

Lactoferrin is a naturally occurring protein ordinarily present in bovine milk. It is historically linked with the production of infant formula,[13] and more recently, it has been used in anti-plaque dental products due to its ability to destabilize the cell membrane of gram-negative bacteria, increasing permeability.[14] It is typically considered safe in oral applications. Endogenously, human Lactoferrin is present in the oral mucosa at low concentrations and in higher concentrations in orally consumed materials such as raw breast milk. [15] Companies that produce bovine lactoferrin have received GRAS approval by the FDA for use in infant formula.[13] An animal study also tested the oral toxicity of Lactoferrin at over 2g/kg/day in rats, and no adverse effects were observed.[16]

There is a strong reason to suspect that Lactoferrin may be efficacious in promoting good oral health and significantly inhibiting the formation of dental plaque.[17] Studies on the Lactoferrin protein have shown that it can protect against Streptococcus mutans-induced caries in mice and reduces the bacterial attachment of *Streptococcus gordonii* in the oral cavity.[18, 19] Finally, Lactoferrin has been used in other clinical studies in combination with lactoperoxidase, and it was found to improve gingival inflammation and supragingival plaque antibacterial effects significantly.[20, 21]

Lactea Therapeutics has formulated a gum product containing an active innate immune protein, MIIP-E2. This product is an ultrapure (>99%) fully native and completely active form of bovine lactoferrin extracted and purified directly from raw milk. MIIP-E2 aims to develop a field-deployable dental care solution to enhance on-the-go oral care targeting military use. Due to the capability of the MIIP-E2 gum to coat the inner surfaces of the mouth, it can function as a barrier to biofilm adherence. The dosage of MIIP-E2 proposed for use in this study is dramatically below these acceptable levels in milk-based infant formulas, which range between 101.3 and 60.1 mg daily.[13]

The primary endpoint of this study is to evaluate the efficacy of MIIP-E2 in inhibiting plaque formation in healthy patients in the absence of tooth brushing using a 4-day plaque accumulation model. The secondary endpoint is to evaluate whether MIIP-E2-containing gum alters the oral microflora after the tested period.

3.0 Objective(s)

3.1 Primary Objective

This study aims to evaluate and compare the efficacy of MIIP-E2 in inhibiting plaque formation between two gum treatments in healthy patients in the absence of tooth brushing using a 4-day plaque accumulation model.

3.2 Secondary Objective

The secondary objective of this study is to evaluate whether MIIP-E2-containing gum alters the oral microflora.

3.3 Tertiary/Exploratory/Correlative Objectives

The exploratory objective is to compare the plaque accumulation between the treatments using photographs.

4.0 Outcome Measures/Endpoints

4.1 Primary Outcome Measures

The primary outcome measures for plaque will be assessed by a dental plaque index, a saliva analysis and intraoral photographs**.**

A. Dental Plaque Assessment: Dental plaque will be identified using a disclosing solution and scored using the Turesky modification of the Quigley and Hein Plaque Index (PLI). (Table 3) Each tooth, except the third molars, will be scored on six sites (Mesio-buccal, buccal, disto-buccal, mesio-lingual, lingual, and disto-lingual). The plaque score index for an individual is determined by adding all the individual scores and dividing the total score by the number of surfaces examined. [22]

B. Salivary analysis for 16S sequencing

In addition, patient saliva samples will be taken before and after each treatment regimen for 16S rRNA microbiome sequencing using the Qiagen PAXgene Saliva Collector and protocols that accompany the collection kit (https://www.qiagen.com /us/products/discovery-and-translational-research/sample-collection-stabilization/dna/pax gene-saliva-collector). Sequencing will be performed at CD Genomics, a third-party vendor, using protocols for full-length PacBio sequencing and

analyzed using standard methodologies to identify microbial taxonomy.[23] No human genetic information will be collected or analyzed using this methodology.

C. Dental Plaque Visual Assessment with photographs Outcome Measure

Images of dental plaque will be used for both qualitative representations of outcomes in future reports as well as used as a secondary, complementary quantitative analysis for the PLI assessment. For the quantitative analysis, plaque coverage on teeth will be assessed using an image thresholding area measurement as previously described. This analysis is anticipated to provide similar results to the PLI as mentioned earlier but may allow for further analysis and quantitative precision. Depending on preliminary analysis, these measurements may be used to further assess details of product efficacy, such as regional changes in plaque across teeth, to understand whether the reduction in plaque occurred primarily on surfaces with direct exposure to the gum or throughout the mouth.[24]

Table 2: Quigley-Hein Plaque Index Scores with Turesky Modifications (PLI)

Score	Description
0	No plaque
1	Separate flecks of plaque at the cervical margin of the tooth
2	A thin continuous band of plaque (up to one mm) at the cervical margin of the tooth
3	A band of plaque wider than one mm but covering less than one-third of the crown of the tooth
4	Plaque covering at least one-third but less than two-thirds of the crown of the tooth
5	Plaque covering two-thirds or more of the crown of the tooth

4.2 Safety Outcome Measure

Safety outcomes will be measured by assessing any study-related soft or hard tissue abnormalities found during the study. Oral soft tissue examinations (OST) will be assessed at the screening visit and at the beginning and the end of each treatment period. Oral Hard Tissue Findings (OHT) will be assessed during the screening and the last subject visits. Any abnormal oral findings will be recorded and evaluated to assess causality and possible relation to the study product. Abnormal findings during the study will be recorded as an AE or SAE.

5.0 Eligibility Criteria

5.1 Inclusion Criteria

- Adult subjects between 18 and 65 years old
- Willing to read and sign the IRB-approved informed consent.
- Healthy, as determined by pertinent medical history at the study dentist's discretion.
- A minimum of 20 natural teeth (excluding third molars) with at least two scorable surfaces per tooth (teeth with full crowns, large/extensive restorations on the interproximal areas, and orthodontic bands will not be included in the tooth count)

- PLI of 2 or greater (Based on the Turesky modification of the Quigley and Hein Plaque Index) calculation based on a whole mouth plaque score.
- Willing to comply with the study procedures.

5.2 Exclusion Criteria

- Presence of any acute or chronic condition, organ system disease, or medication that, in the principal investigator's opinion, could compromise the subjects' ability to participate in the study.
- Gross oral pathologies, including caries, calculus, or soft tissue conditions that show evidence of chronic neglect.
- Evidence of acute periodontal conditions or periodontitis with pockets greater than 5 mm on more than one site
- Use of antibiotics 30 days prior to or during the study
- Requiring the need for antibiotic premedication prior to dental procedures
- Sensitivity to bovine products (lactoferrin) or any of the listed inactive ingredients
- Orthodontic appliances or any removable, except lingual bar retainers
- Self-reported pregnant, wanting to get pregnant, or breast-feeding female,
- Self-reported allergy to disclosing solution ingredients (red dye #28)
- Acute Temporomandibular Disorders (TMD)
- Subject who has participated in other studies (including non-medicinal studies) involving product(s) within 30 days before study screening.
- Subject who has previously been randomized in this study
- An employee of the study site directly involved with the study

6.0 Study Design

This will be a single-blind, single-center, 3-way crossover, randomized controlled clinical trial with 3 treatment periods utilizing 32 subjects. (Fig 1) Each treatment period will be 4 days (96 hours +/—3 hours) in accordance with the plaque accumulation model [25] using one of the following treatment groups: an active compound MIIP-E2 gum (experimental regimen), an inactive compound gum (negative control regimen), and a no-gum (control regimen) . Each subject will be assigned to one of the following three treatment regimen sequences:

1. Control regimen followed by experimental regimen, followed by a negative control regimen, or
2. Experimental regimen followed by negative control regimen, followed by control regimen or
3. Negative control regimen followed by control regimen, followed by an experimental regimen

Subjects will be asked to refrain from oral hygiene (OH) procedures and chewing gum 12 hours (+/- 2 hours) and from eating or drinking (except for water) 2 hours prior to the screening visit. During the 4-day study treatment periods, subjects will also be asked to refrain from oral hygiene practices or any elective dental treatment. Subjects can resume their regular oral hygiene procedures during the wash-out periods of approximately 10 days.

At the screening, potential subjects will be given the IRB-approved informed consent form to read and ask questions. Also, adequate time to decide about their participation will be provided. A study representative trained and delegated by the Principal Investigator will review the IRB-approved consent and answer any questions the potential subject might have before the subject signs/dates the consent. After the subject signs and dates the consent, the study representative will sign and date the consent to confirm that the consent process was completed before initiating any study procedures. The subject will be given a copy of the signed/dated consent.

A designated staff member will collect the medical and concomitant medication information for the study dentist's review. The dentist will perform an oral examination and a PLI to assess the subject's whole-mouth plaque accumulation. A score of 2 or greater will be needed to comply with the study inclusion criteria based on the Turesky modification of the Quigley and Hein Plaque Index (PLI). Other dental characteristics specified on the inclusion/ exclusion criteria will be assessed to determine study eligibility. Eligible subjects will receive a dental prophylaxis and be scheduled for a baseline appointment.

During the baseline appointment, the subjects' continuance criteria and OST examination will be assessed, and an unstimulated saliva sample will be collected. Subjects' teeth will be polished, flossed, and subsequently stained to evaluate total plaque removal and an oral assessment will be performed to confirm the complete removal of plaque. If any plaque is found, the plaque will be removed by either flossing, repolishing the surface, or with an instrument. The dental examiner will assess PLI. Intraoral photos will be taken, including one front and two lateral images (right and left side). 32 subjects will be enrolled and randomly assigned to one of the treatment regimens. Subjects assigned to experimental and negative treatment regimens will perform the first product use under supervision.

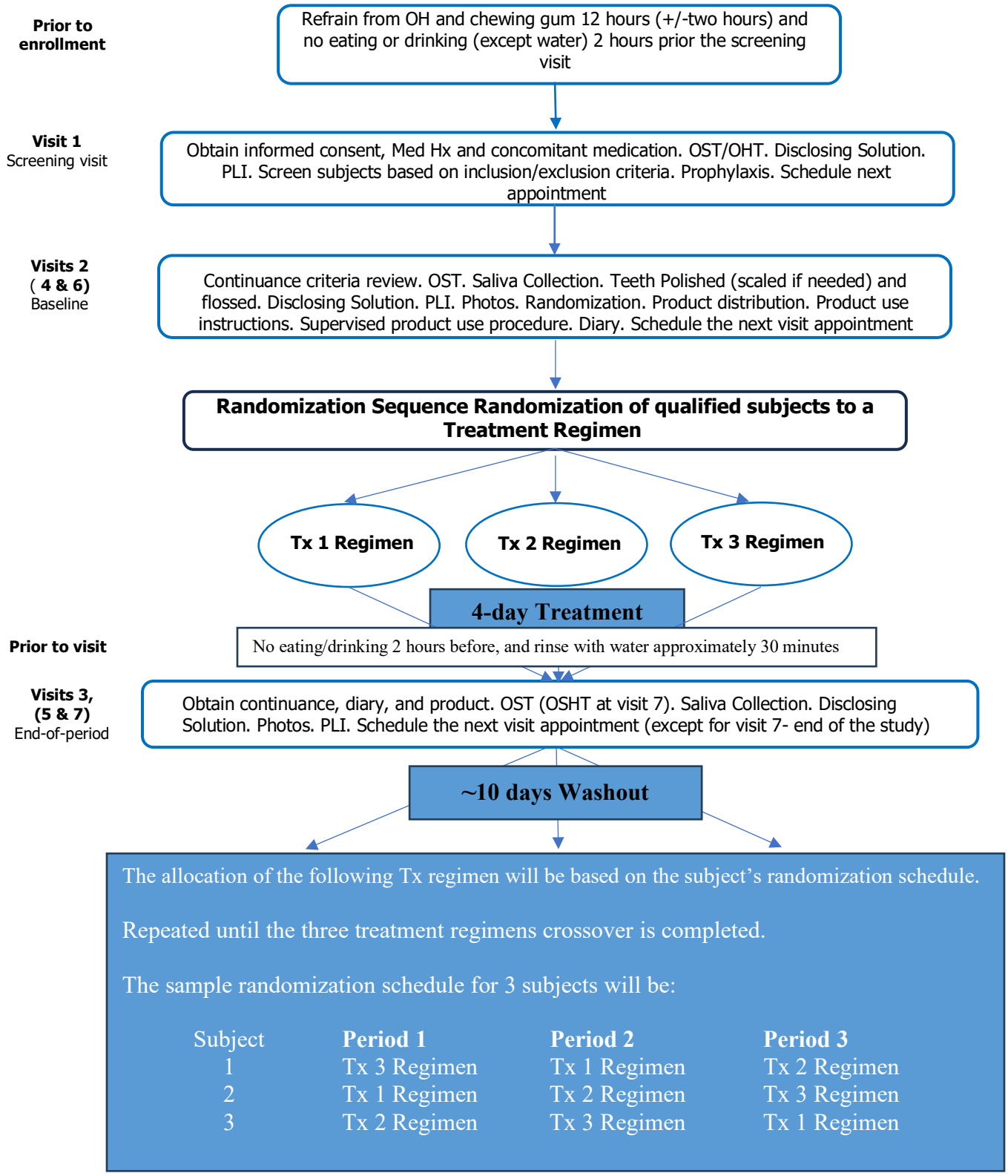
Each subject will be instructed to refrain from using any oral dental hygiene procedures during the study treatment period (4 days). Subjects assigned to the experimental and negative treatment regimen will use only the provided gum product three times a day for 10 minutes after meals (breakfast, lunch, and dinner). Starting on one side of the mouth, chewing for 1 minute, and switching to the other side for another minute. Afterward, they continue chewing for the remaining time however they like. To assess compliance, each subject assigned to a chewing gum product will be provided a diary to record every time they use the product and record any events associated with their study participation. Finally, subjects will be scheduled to return for an end-of-regimen visit.

The last product-use treatment will be after dinner the day before the visit. Subjects will refrain from eating or drinking (except water) for two hours before their appointment. They will also be asked to rinse their mouths with water approximately 30 minutes before their visit.

During the end of the treatment visit, subjects' continuance criteria will be assessed; the diary will be reviewed for compliance; an OST exam will be performed; and an unstimulated saliva sample will be collected. Subjects will rinse with a disclosing solution to reveal the accumulation of dental plaque. Intraoral photos, including one front and two lateral images (right and left sides), will be taken. Afterward, the examiner will assess the PLI, and subjects will be taken to a brushing station to brush their teeth using a provided toothbrush, fluoride-containing toothpaste, and dental floss. They will be scheduled for the next visit after a washout period of approximately 10 days.

For treatments 2 and 3, the procedures mentioned in the baseline visit will be repeated (except for randomization). A new product (or no product) will be given according to the randomization assignment. The end of the treatment visit will be repeated on the fourth day of each treatment period.

Study Flow Diagram (Fig. 1)
3-treatment 3-period Crossover Design



7.0 Enrollment/Randomization

Subjects who previously participated in other Oral Health Research Institute studies (OHRI) or who have contacted OHRI expressing interest in potential studies will be contacted by a team member who will conduct a screening phone interview. If the potential subjects appear to qualify, they will be scheduled for the screening visit.

A sufficient number of subjects will be screened to participate, and 32 will be randomized to finish with at least 30 subjects. We intend to randomize a nearly equal number of female and male subjects. A unique screening number will identify all subjects screened for study participation. Screening numbers will be assigned according to appearance at the study site. Subjects who meet all inclusion and exclusion criteria will be randomized into the study. Block randomization will be used to assign subjects to one of the three treatment regimen sequences:

1. Control regimen (Tx 3) followed by experimental regimen (Tx 1), followed by a negative control (Tx 2), or
2. Experimental regimen (Tx 1) followed by negative control regimen (Tx 2), followed by control (Tx 3) or
3. Negative control regimen (Tx 2) followed by control (Tx 3), followed by experimental regimen (Tx 1).

Randomization numbers will be assigned using randomization schedules provided by the IU Department of Biostatistics and Health Data Science statistician.

The clinical examiners will remain blind to the individual subjects' treatment group during the study. If the treatment assignment must be unblinded for a subject, such as in the case of an emergency, the Principal Investigator will provide written documentation of the unblinding request. Otherwise, blinding will not be broken, unless required in the circumstances detailed above, until all subjects have completed the final study visit and the database has been monitored, locked, and approved by the Principal Investigator.

8.0 Study Procedures

1) Visit 1: Screening Visit (about 1 hour to 1 hour and 45 minutes)

Subjects will refrain from any oral hygiene procedures or chewing gum 12 hours (+/-two hours) and from eating or drinking (except water) 2 hours before this appointment. Following the consenting procedure and demographic/medical history data collection, the following will be performed:

a. An oral exam (including OST and OHT) will be completed.
b. Subjects will rinse with a disclosing solution, and the whole mouth PLI will be assessed and recorded. A Score of 2 or greater will be needed to qualify.
c. Inclusion/Exclusion criteria will be reviewed to determine eligibility.

d. Subjects that qualify will receive a dental prophylaxis.

e. Subjects that do not qualify will be taken to a brushing station to brush and floss their teeth

f. Subject's payment will be given. If qualified, the subject will be scheduled for a baseline visit.

2) Visit 2: Baseline Period 1 Visit (about 1 hour to 1 hour and 30 minutes)

Subjects will be asked to rinse their mouths with water approximately 30 minutes before the visit.

a. Subjects will be assessed for continuance criteria.
b. The study dentist will perform an OST examination.
c. Unstimulated saliva sample will be collected.
d. The subject's teeth will be polished, flossed, and scaled if needed; and subsequently stained with a disclosing solution (using a cotton swab) to evaluate total plaque removal. If any plaque is found, the plaque will be removed by either flossing, repolishing the surface, or with an instrument.
e. An oral assessment will be performed to confirm the complete removal of plaque and re-apply disclosing solution (using a cotton swab), and whole-mouth PLI will be assessed and recorded and the adverse events will be reviewed.
f. Intraoral photos, including one front and two lateral images (right and left sides), will be taken.
g. Subjects will be randomized to a treatment regimen.
    i. A qualified study team member will prepare and distribute the randomized study product (chewing gum), how-to-use instructions, lifestyle restrictions, and compliance dairy to the subject.

    ii. Subjects assigned to the Tx 3 regimen will not receive any product and will be asked not to brush their teeth during the four-day (96 hours +/—3 hours) study treatment.

    iii. Subjects in the Tx 1 and Tx 2 regimens must use the provided gum for four days (96 hours +/—2 hours), chewing gum after each meal (three times a day—breakfast, lunch, and dinner). They start on one side of the mouth, chew for one minute, and switch to the other side for another minute. After that, they can continue chewing for the remaining time in any way they want.

    iv. All subjects will be asked to refrain from brushing or flossing their teeth during the study regimens and from any professional dental cleaning or dental elective procedures.

h. The subject will be scheduled for an end-of-treatment visit on day 4 (96 hours +/- 3 hours) of treatment. Subject payment for the visit will be given.

3) Visit 3: End of Period 1 visit -scheduled at 96 hours +/- 3 hours (about 1 hour and 10 min)

The last product-use treatment will be after dinner the day before the visit. Subjects will refrain from eating or drinking (except water) for two hours before their appointment. They

will also be asked to rinse their mouths with water approximately 30 minutes before their visit.

a. Subjects will be assessed for continuance criteria, and the diary and product will be collected.
b. The study dentist will perform an OST examination.
c. Unstimulated saliva sample will be collected.
d. Subjects will rinse with a disclosing solution to reveal the dental plaque accumulation and intraoral photos will be taken, including one front and two lateral images (right and left sides).
e. Whole mouth PLI will be assessed and recorded, and subjects will be taken to a brushing station to brush and floss their teeth.
f. Subjects will receive payment for Visit 3 and be scheduled for Visit 4: Baseline treatment 2 after a washout period of approximately 10 days.
g. Subjects can resume habitual oral hygiene procedures during the washout period.

4) **Visits 4-7:** The procedures mentioned above will be repeated until each subject has completed each treatment period. At visit 7, in addition to the procedures described in Visit 3, the subjects will also receive an oral hard tissue examination, and their participation in the study will end.

5) Oral Exam

The study dentist will complete an oral soft and hard tissue (OST and OHT) examination at screening and at the end of the study visits. Oral soft tissue (OST) examination will be assessed at the beginning and the end of each treatment period. The exams will be conducted via a visual examination of the oral cavity and perioral area utilizing a light source, dental mirror, gauze, periodontal probe, and tongue blade, as needed. The soft tissue structures examined will involve the labial mucosa, including lips, buccal mucosa, mucogingival folds, gingival mucosa, hard and soft palate, tonsillar and pharyngeal areas, tongue, sublingual area/floor of the mouth, submandibular area, major salivary glands, head and neck, and TMJ. Observations will be listed as "Normal" and "Abnormal," and abnormalities will be described.

The hard tissue structures examined will include assessing for enamel Irregularities, tooth fracture, pathologic tooth wear, cavitated lesions, residual roots, faulty restorations, and implants. Observations will be listed as "Absent" or "Present," and conditions noted as present will be described.

6) Unstimulated saliva collection

Unstimulated saliva will be collected during each study treatment at the baseline and end of the treatment visits using the PAXgene saliva collector kits. Subjects will rinse their mouth with water, to remove any food debris, approximately 30 minutes prior to their appointment. They will not drink, eat, smoke or chew gum after they rinse their mouths. They will sit quietly for five minutes before beginning the collection. Subjects will spit gently into the provided tube with an attached funnel for about 5 to 10 minutes until the saliva samples (only liquid saliva, not bubble) reach the full line mark indicated in the tube. During this time,

they will be told to allow their saliva to pool, emptying it into a collection funnel whenever they need to swallow.

PAXgene's instructions for use will be followed for post collection. The samples will be stored and shipped to the sponsor at ambient temperature within the week they are collected.

7) Salivary 16S sequence analysis

Salivary samples will be sequenced at a third-party vendor, CD Genomics, where they will perform DNA isolation and use PacBio Full Length 16S rRNA sequencing to maximize taxonomical resolution, as previously reported.[23] Lactea Therapeutics will analyze the relative abundance of each identified microbial community in each sample before and after treatment and in the after-treatment groups for each condition. No human genetic data will be collected from patient samples.

8) Disclosing Solution

During the screening and end of treatment visits, subjects will be seated upright in the dental chair, and lips will be covered with Vaseline to avoid staining. Afterward, they will be asked to swish with 10 cc of a disclosing solution (Trace -Young Dental) for 10 seconds and gently spit the solution-saliva into a Styrofoam cup. The excess Vaseline will be removed from the lips, prior to PLI assessment or taking intraoral photos.

For evaluation of plaque removal after teeth are polished and prior to baseline PLI assessment, a small amount of disclosing solution will be dispensed into a cup then directly applied to the teeth with a cotton swab; the excess of the disclosing solution will be suctioned from the subject's mouth.

9) Intraoral Photographs

Three intraoral photographs (one on the front and two lateral images) will be taken at the beginning and end of each treatment right after the dental plaque is disclosed. No facial features will be captured in the photographs. A digital single-lens reflex camera (Nikon D200) with a 50 mm macro lens and a magnification ratio of 1:1 will be used. The light source will also be an external ring flash mounted in front of the lens. A double-ended cheek retractor will retract the lips and other soft tissue structures that may obstruct the frontal and lateral views to capture a better image of the teeth Intraoral mirrors will be used to take the lateral images (views).

Subjects will be seated upright in the dental chair, with the head in a neutral position, keeping the occlusal plane perpendicular to the floor and the teeth in a maximum intercuspation position. For the frontal shot, the subject will hold the cheek retractors, and the camera will be placed at a 90-degree angle from the front teeth, focusing only on the anterior teeth (canine to canine). The lateral images will be captured using an intraoral mirror, and the retractors will be used similarly, capturing the buccal images of the premolar and molar teeth.

10) Photograph Analysis

The sponsor may perform the analysis of these photographs in a future time. The analysis is planned to be performed by measuring the plaque surface of each tooth using Adobe Photoshop. The dental plaque present will be outlined using the "Pen Tool" and" Make Path"

tools. Specific settings for the "color range" option will be used to highlight the disclosed plaque surfaces. The areas not covered with plaque will be identified using the "Mask" option. The plaque image will be transferred to a new file, and it will be analyzed using Image Pro Plus software, which will provide a measurement of the total area in mm^2.[24]

11) Dental Prophylaxis

This procedure will be performed before the first treatment assignment. It will include scaling any tartar or dental plaque present on the crown of the teeth using dental scalers or ultrasonic instruments if necessary. After scaling, the teeth will be polished using very fine pumice without fluoride and flossed. A qualified licensed hygienist will perform this procedure under the supervision of the study dentist.

12) Teeth Polishing and Flossing

This procedure will be performed at the beginning of each treatment. It will include removing any plaque deposits by polishing the teeth with very fine pumice without fluoride and flossing. A scaler instrument could be used if needed. A qualified licensed hygienist will perform this procedure under the supervision of the study dentist.

13) Treatment Compliance

Product use compliance will be assessed by counting the number of gum pieces before dispensing (24 pieces) and at the end of each treatment period. Subjects will be required to return all products left over to the study site, and the number of gum pieces will be recorded on the treatment dispensing log.

14) Plaque Index (PLI)

Supragingival plaque will be assessed following disclosing with the Trace disclosing solution. According to the criteria of the Turesky modification of the Quigley-Hein Plaque Index (Table 2). Each tooth will be visually assessed on six surfaces (Mesio-buccal, buccal, disto-buccal, mesio-lingual, lingual and disto-lingual). For PLI calculation, all the individual scores will be added and divided by the number of surfaces examined. The same trained examiner will also perform this assessment. (Table 2)

15) Home Use Diary

At the start of the study period, subjects assigned to the experimental and the negative control treatment will be provided with a diary to record the date and time of the morning (a.m.), afternoon (p.m.), and evening (p.m.) gum chewing procedures and any deviation from the regimen. Subjects assigned to the control regimen a diary will provided to record no oral dental hygiene procedures performed including chewing gum during the treatment period. In addition, subjects will record any new or changes in pre-existing medical conditions, medications, or treatments or any change in signs or symptoms.

Subjects must bring the completed diary to the end of the treatment visit. Study staff will review the diary with the subject to confirm treatment compliance and clarify listed medical conditions, medications, and treatments. Subjects assigned to the experimental and the negative control can miss up to 10% of their chewing times (1 missing chewing per period) during the duration of each study period. Those subjects who miss more than one chewing time will be considered non-compliant, and a staff member will instruct them about the

importance of keeping chewing times in compliance or remove them from future treatment groups if necessary.

16) Active compound containing gum

The active compound containing gum or MIIP-E2 gum V1 is comprised of Sorbitol, Gum Base, Mannitol, Xylitol, Triacetin, natural peppermint flavor, wax, MIIP-E2, Liquid flavoring (with coconut oil), sucralose, BHT, silicon dioxide and magnesium stearate. Each piece of gum is approximately 2.4 g in weight and contains 30 mg of the active ingredient, MIIP-E2. For this study, the gum containing the active ingredient will be colored purple.

17) Non-active compound containing gum

The non-active compound containing gum is comprised of Sorbitol, Gum Base, Mannitol, Xylitol, Triacetin, natural peppermint flavor, wax, Liquid flavoring (with coconut oil), sucralose, BHT, silicon dioxide, and magnesium stearate. Each piece of gum is approximately 2.4g. For this study, these gum pieces will be colored white.

18) Lifestyle Requirements

Subjects will refrain from brushing their teeth and chewing gum 12 hours (+/- two hours) prior and not eating or drinking anything (except water) 2 hours before oral assessments at the screening visit. They will also refrain from using any oral hygiene procedures during the treatment days, except for using the provided gum if they have the product assigned.

Subjects in the Tx1 and Tx2 regimens must use the chewing gum provided after each meal (three times a day—breakfast, lunch, and dinner). They should start chewing on one side of the mouth, chew for one minute, and switch to the other side for another minute. After that, they can continue chewing the remaining time (8 minutes) in any way they want. The last product-use treatment will be after dinner the day before the end of the treatment visit. Subjects will refrain from eating or drinking (except water) for two hours before their appointment. They will also be asked to rinse their mouths with water approximately 30 minutes before their visit.

During the 4-day study period, subjects will refrain from brushing, flossing, chewing gum, or having any elective dental procedures.

9.0 Reportable Events

Adverse events are not anticipated with the MIIP-E2 or control gum or its packaging. The product is designed to break easily if too much pressure is applied. Cheek bites are anticipated as possible events associated to chewing gum. No toxicity is anticipated for the active compound. However, any tissue changes during the study will be documented and reported. The product is packaged in a small clear plastic bag that is easy to open and close.

Subjects will be questioned regarding any general health or oral complaints and symptoms they have experienced during or following their treatment. Any findings will be documented on the AE CRF. Subjects reporting AEs outside the scheduled clinical visit will be assessed by the study dentist and/or principal investigator as soon as possible. All AEs, regardless of severity or relationship to the treatment product, will be recorded.

Serious AEs include any events resulting in death, decreased life expectancy, life-threatening situations, persistent or permanent disability/incapacity, hospitalization, or congenital anomaly/congenital disability. Within 24 hours, the Principal Investigator will submit a written report documenting the circumstances of the serious AE. The IRB will be notified within five days of the incident.

10.0 Data Safety Monitoring

The Principal Investigator and the entire study team will monitor the data and safety. They will monitor data quality, subject recruitment, accrual, retention, outcome, and adverse event data, or assessment of scientific reports, results of related studies that may impact subject safety, and procedures designed to protect subjects' privacy. Safety data will be monitored regularly and immediately upon discovery of any SAE, major study event, or protocol deviation.

11.0 Study Withdrawal/Discontinuation

Subjects may withdraw from the study for any reason and at any time during the study without penalty. Their decision to stop study-related procedures will not affect their dental treatment. The Principal Investigator may also withdraw the subject if they feel study participation is a safety concern or the subject is not compliant with study procedures.

12.0 Statistical Considerations

Statistical comparisons will be performed among all three treatment regimens. All outcome variables will be analyzed using a mixed-model analysis of variance suitable for a crossover design. The model will include a random effect for each subject to account for correlations among treatment outcomes within a subject, and fixed effects for study period and treatment regimen. A treatment sequence effect will be examined as an indicator of a carryover effect and removed from the model if not significant. Distributions of the data will be evaluated and a transformation of the data (e.g., logarithmic) or nonparametric tests will be used if needed. A false discovery rate (FDR) adjustment will be used to account for the large number of tests in the oral microbiome analyses. A two-sided 5% significance level will be used for all tests.

32 subjects will be enrolled in the study to ensure 30 complete the study. Based on a previous study, the estimated PLI treated mean is 1.8, standard deviation for PLI is 0.6, and within-subject correlation is 0.75, for an estimated standard deviation of differences 0.42. The study will have 80% power at a two-sided 5% significance level to detect a difference of 0.22 between two treatment regimens (12% relative difference), based on a paired t-test calculation.

13.0 Statistical Data Management

Primary data will be collected via paper source documents and CRF's will be electronically stored as an excel document on the OHRI internal drive. The storage location will be backed up automatically every day. Quality assurance steps will follow the IU UITS service management SOPs.

14.0 Privacy/Confidentiality Issues

To maintain privacy, discussions about the study, the consenting process, and all other study procedures will be conducted in a dental office away from public places.

Participants are asked to not comment on social media or electronic platforms about the study and refrain from discussing the study outside of their family members/guardians/caretakes, medical professionals, or study staff.
Paper records will be kept confidential and stored in a locked area only study personnel can access. Subjects will be given a study number that will identify all documents. Documents containing the subject's name (like the signed consent form) will be stored separately from other study documents.

Electronic data and photographs will be stored in an encrypted, password-protected computer file that only study personnel can access.

Photographs will be shared with the sponsor for later analysis, and the photos will not have any identifying futures except for the subject number and the leg and visit when the photos were taken. Examples: L1001BL/ L1001EL

15.0 Follow-up and Record Retention

Study records will be maintained for at least seven years, as per Indiana law.

16.0 References

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