Study Protocol (Public Version)

Microbiological and Clinical Effects of Pre- and Probiotics in Non-Surgical Periodontal Therapy (PROPARO)
Protocol ID: **S-477/2024**
Sponsor/PI: **Heidelberg University Hospital, Department of Conservative Dentistry (Poliklinik für Zahnerhaltungskunde)** / **Prof. Dr. Diana Wolff** (Study Lead)
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Study Site(s): **Single-center (Heidelberg University Hospital, Germany)**
Planned Duration: **24 months (recruitment + follow-up)**

1. Background and Rationale

Periodontitis is a common chronic inflammatory disease characterized by periodontal pocketing, bleeding on probing, and alveolar bone loss, driven by a dysbiotic biofilm and a dysregulated host response. Standard non-surgical periodontal therapy (NSPT) is primarily mechanical and may be complemented by antiseptics or systemic antibiotics in selected cases. However, routine "microbiome engineering" is not established due to limited high-quality evidence.

Probiotics (live microorganisms conferring a host benefit when administered in adequate amounts) have been investigated as adjuncts to NSPT, with some evidence suggesting improvements in clinical parameters and/or shifts in periodontal pathogens, particularly for *Limosilactobacillus reuteri*. Prebiotics are substrates selectively utilized by microorganisms that may confer a competitive advantage. Vitamin B12 has been discussed as potentially supporting specific microbial metabolic functions relevant to *L. reuteri*. To date, the combined adjunctive use of a probiotic and a prebiotic-acting supplement in NSPT has not been adequately studied with comprehensive microbiome endpoints.

2. Objectives and Hypothesis

2.1 Primary Objective

To evaluate whether adjunctive oral supplementation with a probiotic (*L. reuteri*)—alone or combined with vitamin B12—modifies the **composition and structure of the supragingival and subgingival oral microbiome** during NSPT.

2.2 Secondary Objectives

To compare groups with respect to:

- Periodontal and oral clinical status and inflammatory indicators during standard care
- Patient-level ecological covariates relevant to microbiome composition (diet quality, perceived stress, physical activity)
- Feasibility endpoints (recruitment, adherence, completeness of biospecimen collection)

2.3 Hypothesis

Combined supplementation (probiotic + vitamin B12) will produce a **more favorable and sustained shift** of a dysbiotic microbiome toward a more balanced state compared with probiotic alone and compared with standard care without supplementation.

3. Study Design

- **Type:** Single-center, randomized, controlled **pilot** study
- **Allocation:** 1:1:1
- **Masking: Single-blind** (clinical examiner blinded to group allocation where feasible); participants not blinded (different dosage forms)
- **Control:** Standard care without placebo
- **Framework:** Superiority (exploratory/pilot; hypothesis-generating)

4. Participants

4.1 Target Population

Adults with **diagnosed periodontitis (localized or generalized), Stage III or IV**, scheduled for NSPT.

4.2 Inclusion Criteria

- Age **≥ 18 years**
- Stage III or IV periodontitis (localized or generalized)
- Ability to provide informed consent
- Written informed consent including data protection consent

4.3 Exclusion Criteria (key)

- Pregnancy or breastfeeding
- Conditions/medications likely to substantially confound the oral microbiome or periodontal healing (e.g., uncontrolled diabetes mellitus, systemic immunosuppression, chronic steroid use)
- Antibiotic use within the last **3 months**
- Use of oral pre-/probiotic supplements within the last **3 months**
- Recent subgingival instrumentation within the last **6 months**
- Need for obligatory adjunctive systemic antibiotic prophylaxis/therapy for NSPT
- Severe active systemic infection or other conditions judged by investigators to pose undue risk
- Regular use of antiseptic mouthrinses during the study period (unless medically required)
- Strict diets likely to substantially alter baseline microbiome trajectories (e.g., ketogenic, strict vegan) per protocol definition
- Chronic bowel diseases (for systemic confounding considerations)

5. Interventions

All groups receive **guideline-concordant non-surgical periodontal therapy (standard care)** including oral hygiene instruction and supportive periodontal therapy (SPT/UPT) follow-up.

5.1 Study Arms

Group 1 (Control): Standard NSPT/SPT, **no** pre-/probiotic supplementation.

Group 2 (Test A – Probiotic): Standard NSPT/SPT **plus** probiotic lozenge containing *L. reuteri* (commercial dietary supplement; **GUM® PerioBalance®**, Sunstar Deutschland GmbH, Schönau, Germany).

- Dosing and duration: **According to manufacturer instructions**, initiated in conjunction with NSPT and continued for **3 months**.

Group 3 (Test B – Synbiotic-like: Probiotic + Vitamin B12): Standard NSPT/SPT **plus** the same probiotic regimen **and**vitamin B12 drops (commercial dietary supplement; **natural elements GmbH**, Düsseldorf, Germany).

- Vitamin B12 dosing: **According to manufacturer instructions** (planned daily intake corresponds to 500 µg).
- Duration: **3 months**, aligned with probiotic regimen.

Re-administration during SPT/UPT: If clinically indicated subgingival re-instrumentation is performed within routine supportive care, the assigned supplementation regimen may be repeated **as per protocol-defined standardization**.

5.2 Concomitant Care and Restrictions

- Standardized toothpaste provided for home use
- Participants follow individualized oral hygiene instructions
- Avoidance of additional antimicrobial adjuncts (e.g., antiseptic mouthrinses) unless clinically required and documented

6. Outcomes

6.1 Primary Outcome

Oral microbiome composition (supragingival and subgingival):

- Global community structure and diversity (alpha/beta diversity measures)
- Relative abundance of taxa/features and functional profiles (as available)

6.2 Secondary Outcomes

- Clinical periodontal parameters: PD, CAL, BOP, furcation involvement, mobility grades
- Oral hygiene/gingival indices: Plaque Control Record (O'Leary), Gingival Bleeding Index (Ainamo & Bay)

- Dental status: DMFT
- Patient-reported covariates:
    - Diet quality (DEGS nutrition questionnaire; MEDAS)
    - Perceived stress (PSS)
    - Physical activity (IPAQ)
    - Demographic and socioeconomic information

6.3 Safety Outcomes

- Adverse events (AEs) and serious adverse events (SAEs), with particular attention to intolerance to supplements and any unexpected oral/systemic events.

7. Study Procedures and Schedule

7.1 Study Visits / Time Points

Biospecimens and clinical measures will be collected at **four** standardized time points aligned with clinical care:

1. **Baseline / NSPT start**
2. **Day of full-mouth instrumentation (FMS, per clinic routine)**
3. **Re-evaluation / first SPT visit (~3 months)**
4. **Subsequent SPT visits (~6 and ~12 months)** (as scheduled within standard care)

(Exact visit windows and operational timing are specified in the internal operations manual.)

7.2 Biospecimens

- **Supragingival plaque**
- **Subgingival plaque**
- **Unstimulated saliva**

All collections use **standard, routine dental sampling approaches**. Detailed collection mechanics (materials, handling, timing, storage logistics) are maintained in **internal SOPs**.

8. Laboratory and Bioinformatics (High-level)

8.1 Molecular Methods

- **16S rDNA-based profiling** for community composition
- **Shotgun metagenomic sequencing** for species-level resolution and functional inference (where applicable)

8.2 Data Processing (High-level)

- Standard quality control, taxonomic assignment, and diversity metrics using validated microbiome workflows
- Differential abundance and multivariable modeling to evaluate group effects while accounting for relevant covariates

(Parameter-level pipeline specifications, QC thresholds, and code repositories are retained internally and can be shared upon reasonable scientific request after primary publication, consistent with institutional policy.)

9. Sample Size

This is a **pilot feasibility** study. Because robust prior estimates for microbiome change under this specific intervention combination are unavailable, a formal power calculation is not feasible. For feasibility and precision-of-estimate purposes, **n = 60** participants are planned (**20 per arm**).

10. Randomization and Allocation

- **Method:** Stratified block randomization
- **Stratification variables:** Sex, age category, and periodontitis diagnosis category
- **Allocation concealment:** Implemented via a pre-generated randomization list managed by authorized study personnel not involved in outcome assessment.

11. Blinding

- The **clinical examiner** assessing periodontal endpoints is intended to be **blinded** to group allocation where operationally feasible.
- Participants and dispensing personnel are **not blinded** due to distinguishable supplement forms and the absence of a placebo.

12. Statistical Analysis Plan (Public Summary)

12.1 Primary Analysis

- Between-group comparisons of microbiome community structure over time using:
    - Diversity metrics (alpha/beta)
    - Ordination-based visualization (e.g., PCoA)
    - Permutational multivariate methods (e.g., PERMANOVA) for group/time effects
- Differential abundance analyses and multivariable models to assess intervention effects while adjusting for relevant covariates (diet, stress, physical activity, smoking status).

12.2 Secondary Analyses

- Descriptive statistics for clinical outcomes and patient-reported variables
- Exploratory comparisons of clinical periodontal measures between groups over time (appropriate longitudinal models)

12.3 Handling of Missing Data

- Completeness of biospecimens and questionnaires will be summarized
- Analyses will use appropriate methods for missingness consistent with pilot-study reporting

(A detailed internal SAP exists separately.)

13. Safety Monitoring

- Participants are monitored for AEs/SAEs at each study contact.
- Supplements used are marketed as dietary supplements in Germany; nevertheless, any suspected supplement-related intolerance or adverse reactions will be documented and managed according to clinical judgment and institutional procedures.

14. Ethical Considerations and Data Protection

- Conducted in accordance with the **Declaration of Helsinki** and applicable German/EU regulations.
- Ethics approval will be obtained from the **Ethics Committee of the Heidelberg Medical Faculty** before study start.
- Written informed consent is required.
- Data will be **pseudonymized**; access to re-identification codes is restricted to the study lead.
- Data and biospecimens will be stored for up to **15 years after publication**, consistent with institutional policy and applicable law.
- Participants may withdraw at any time without disadvantages; handling of already collected data/material follows the consent form provisions.

15. Study Status and Dissemination

- Recruitment begins after ethics approval and site readiness.
- Results will be disseminated via peer-reviewed publication and conference presentations.
- Trial registration will be completed prior to enrollment; primary and key secondary outcomes will be specified prospectively to prevent outcome switching.

16. Conflicts of Interest and Funding

- Conflicts of interest: **None declared** (update if applicable)
- Funding/support: **[insert]** (e.g., institutional resources; clarify if any in-kind supplement provision)