Protocol

Impact of a Personalized Preventive Strategy on Oral Microbial Homeostasis During Fixed Orthodontic Treatment (PREPERMIO): Protocol for a Randomized Controlled Trial

Margaux Dubois[1,2], DDS, PhD; Morgane Ortis[1], PhD; Lilit Tonoyan[1], PhD; Alain Doglio[1], PhD; Marie-France Bertrand[1,2], DDS, PhD; Olivier Sorel[3], DDS, PhD; Laurence Lupi[1,2], DDS, PhD

[1]Laboratoire MICORALIS, Faculté de Chirurgie Dentaire Odontologie, Université Côte d'Azur, Nice, France

[2]Institut de Médecine Bucco-Dentaire, Centre Hospitalier Universitaire de Nice, Nice, France

[3]Private Practice in Orthodontics, Dinan, France

Corresponding Author:
Laurence Lupi, DDS, PhD
Laboratoire MICORALIS, Faculté de Chirurgie Dentaire Odontologie
Université Côte d'Azur
5 Rue du 22ème BCA
Campus Saint Jean D'Angely, Bâtiment SJ2
Nice, 06300
France
Phone: 33 616195088
Email: Laurence.LUPI@univ-cotedazur.fr

Abstract

Background: Fixed orthodontic appliances create plaque-retentive niches that promote dental biofilm accumulation and are associated with enamel demineralization, periodontal inflammation, and ecological shifts in the oral microbiota. Previous orthodontic prevention studies have mainly focused on behavioral or clinical outcomes, with limited integration of longitudinal microbiological and host inflammatory assessments. Advances in saliva-based microbial and immunological profiling now provide opportunities to better characterize treatment-associated dysbiosis and related host inflammatory responses and to support more individualized preventive approaches during orthodontic care.

Objective: This study aims to evaluate whether an intensive personalized prevention strategy, added to standard orthodontic care, can limit orthodontic treatment–associated microbial dysbiosis during the first year of fixed orthodontic treatment compared with standard care alone.

Methods: PREPERMIO (Personalized Prevention During Fixed Orthodontic Treatment and Oral Microbial Homeostasis) is a prospective, multicenter, randomized, open-label, parallel-arm interventional study conducted in France. A total of 80 participants aged 12-20 years requiring fixed orthodontic treatment will be randomized 1:1 to receive either standard care or standard care plus personalized prevention. The preventive intervention includes repeated individualized oral health education, supervised toothbrushing, dietary counseling, plaque disclosure, and standardized chairside prophylaxis adapted according to each participant's clinical and behavioral profile. Participants will be followed for 12 months, with assessments performed at baseline and at 3, 6, 9, and 12 months. The primary outcome is longitudinal changes in predefined microbial ecological patterns in saliva and dental biofilm, reflecting dysbiosis over time, assessed using high-throughput microfluidic quantitative polymerase chain reaction for selected bacterial and viral targets. Dysbiosis is defined as temporal compositional and quantitative shifts in the relative and absolute abundance of commensal and pathogenic microbial species within the oral microbial community. Secondary outcomes include salivary inflammatory profiling (cytokines/chemokines), clinical indices (plaque index, bleeding on probing, or white spot lesions), compliance measures, and participant satisfaction. Longitudinal analyses will primarily rely on mixed effects models.

Results: The study received ethics approval from the French Committee for the Protection of Persons (CPP 2024-A00646-41) in December 2024 and was notified to the French National Agency for the Safety of Medicines and Health Products in December 2024. Participant recruitment had not yet started at the time of protocol submission. Recruitment is planned between March and September 2026, with follow-up continuing until September 2027. Database lock, statistical analyses, and dissemination of results are expected in early 2028.

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Conclusions: PREPERMIO is a randomized interventional orthodontic study evaluating a personalized preventive strategy using biologically driven longitudinal end points integrating bacterial, viral, inflammatory, clinical, and behavioral dimensions. By focusing on microbial ecological shifts during fixed orthodontic treatment, this protocol may contribute to the development of more biologically informed and personalized preventive approaches in orthodontic care.

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KEYWORDS

cytokines; dysbiosis; microbiota; oral hygiene; orthodontic brackets; primary prevention; randomized controlled trial

Introduction

Background and Rationale

The human oral cavity hosts a complex and dynamic microbiota composed of more than 700 bacterial species, organized either as planktonic communities in saliva or as structured biofilms adhering to hard and soft oral tissues [1]. Oral biofilms are structured microbial communities whose composition and pathogenic potential are influenced by environmental factors and host-related determinants, including salivary factors, immune responses, and oral hygiene practices [1-4]. Under physiological conditions, a balanced host-microbiota interaction contributes to oral homeostasis. However, this fragile equilibrium is easily disrupted, leading to a dysbiotic biofilm accumulation and maturation, which play a central etiopathogenic role in the development of the 2 most prevalent oral diseases worldwide: dental caries and periodontal diseases [5-7]. Furthermore, dysbiotic alterations in biofilm composition have been consistently associated with the activation of host inflammatory pathways [8-10].

Fixed orthodontic appliances profoundly reshape the oral ecological environment by increasing plaque-retentive surfaces and compromising effective mechanical biofilm removal. These ecological changes promote dental biofilm accumulation and are associated with enamel demineralization, gingival inflammation, and shifts in the oral microbial ecosystem [11-13]. Orthodontic treatment has been shown to induce both qualitative and quantitative changes in the oral microbiota [14]. Increasing evidence suggests that it may induce progressive alterations in microbial community composition, characterized by shifts in the balance between health-associated commensal microorganisms and disease-associated pathogenic species [15]. Several studies have reported microbiological changes during fixed orthodontic treatment, including increased abundance of cariogenic streptococci and periodontal pathogens [14,16,17]. More recent longitudinal microbiome investigations have confirmed dynamic shifts in microbial composition and inflammatory responses throughout orthodontic treatment, emphasizing substantial interindividual variability in ecological trajectories [18-21].

Over the past decade, advances in molecular taxonomy, high-throughput sequencing, and saliva-based multiomics approaches have enabled more detailed characterization of oral microbiota and host-derived biomarkers [22,23]. Salivary biomarkers, including microbial and inflammatory markers, reflect both local ecological changes and host response, and may provide noninvasive and clinically relevant indicators of disease risk [24-26]. However, existing studies are heterogeneous in terms of design, follow-up duration, sampling strategies, and outcome measures. Many focus predominantly on clinical indices or short-term microbiological assessments, limiting their ability to capture longitudinal ecological changes in the oral ecosystem.

As ecological disruption and microbial dysbiosis are increasingly recognized as central mechanisms in the pathogenesis of oral diseases, attention has turned toward preventive approaches aimed at maintaining microbial homeostasis. Recent translational microbiome studies support the emergence of biologically informed and microbiome-oriented preventive strategies in oral health care [21]. Despite growing recognition of the ecological mechanisms underlying treatment-related oral complications, preventive strategies during orthodontic treatment mainly rely on standardized oral hygiene instructions and periodic clinical monitoring [27,28]. While some studies have investigated microbiological and inflammatory changes during orthodontic treatment [29-31], many prevention-focused studies still primarily assess clinical outcomes such as plaque accumulation or white spot lesions and/or behavioral adherence, with fewer integrating longitudinal microbial and host inflammatory trajectories in an interventional context. Identifying combined microbial and inflammatory biological signatures could allow early identification of individuals at increased risk of adverse oral outcomes during fixed orthodontic treatment [32]. However, longitudinal studies integrating microbial profiling and salivary biomarkers in orthodontic preventive settings remain limited [33-35]. Addressing this gap may support the development of risk-stratified, personalized preventive strategies, beyond uniform recommendations, to improve oral health outcomes during orthodontic care.

This protocol, referred to as PREPERMIO (Personalized Prevention During Fixed Orthodontic Treatment and Oral Microbial Homeostasis), was designed as a biologically driven randomized interventional study evaluating whether an intensive personalized preventive strategy can limit treatment-associated microbial ecological shifts and improve oral health outcomes during fixed orthodontic treatment, while also exploring longitudinal microbial and inflammatory patterns potentially associated with treatment-related dysbiosis.

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Study Hypothesis

Fixed orthodontic treatment is associated with the development of oral microbial dysbiosis [12,34,35], due in part to increased plaque-retentive sites and reduced effectiveness of routine oral hygiene measures, even in patients with good hygiene practices. Building on this evidence, we hypothesize that oral dysbiosis develops progressively during fixed orthodontic treatment, as evidenced by qualitative alterations in the microbial profile toward a more dysbiotic state, accompanied by quantitative shifts in the abundance of selected commensal and pathogenic bacterial species, and that an intensive, personalized prevention strategy is more effective than standard care alone in limiting or preventing this dysbiosis. To test this hypothesis, 2 groups of patients undergoing fixed orthodontic treatment will be compared: one receiving standard preventive care and one receiving a personalized prevention strategy based on individual biological profiles. We further hypothesize that the integration of microbiological and immunological markers may provide exploratory insights into individual risk profiles and support a more targeted, patient-specific preventive approach during orthodontic treatment.

Aims and Objectives

The primary objective of this study is to compare the longitudinal changes in oral microbiota composition and abundance in saliva and dental biofilm between two preventive strategies: (1) standard preventive care alone and (2) standard care supplemented with an individualized preventive intervention with the aim of evaluating whether the personalized preventive strategy can limit treatment-associated microbial ecological shifts during fixed orthodontic treatment. Microbiological assessments will be performed at baseline, corresponding to the day of appliance placement, and at 3, 6, 9, and 12 months of follow-up.

The secondary objectives are to compare the two groups at the same time points with respect to (1) host immune response, assessed through salivary immune markers; (2) clinical oral outcomes, including plaque accumulation, gingival inflammation, and enamel demineralization (white spot lesions); (3) patient compliance with oral hygiene and preventive measures; and (4) patient satisfaction with preventive care during orthodontic treatment.

In addition, exploratory analyses will investigate microbial and inflammatory signatures potentially associated with increased susceptibility to dysbiosis.

Methods

Study Design and Setting

This study is a prospective, multicenter, randomized, open-label, parallel-arm interventional trial with a 12-month follow-up. Participants will be enrolled at two sites (multicenter) in France: (1) the Institute of Oral Medicine at Nice University Hospital (CHU Nice; public investigative center) and (2) a private orthodontic practice in Dinan. They will be followed forward in time (prospective) and randomly assigned (randomized) in a 1:1 ratio to either a control group receiving standard preventive care or an intervention group receiving standard care combined with a personalized prevention strategy (interventional). Both participants and clinicians will be aware of group allocation (open-label). The 2 groups will be followed concurrently (parallel-arm), allowing direct comparison between standard care and standard care plus personalized prevention.

This study was designed by the research team without direct involvement of patients or the public in the planning, conduct, or reporting stages.

Participants, Interventions, and Outcomes

Eligibility Criteria

Textbox 1 presents the eligibility criteria for participants.

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Textbox 1. Eligibility criteria.

Inclusion criteria

- Aged 12-20 years, with permanent dentition
- Covered by the French social security system
- Indicated for fixed orthodontic treatment following a comprehensive orthodontic assessment
- Willing to participate, documented by written informed consent; for participants aged <18 years, written informed consent will be obtained from both parents or from the legal guardians holding parental authority, in accordance with French regulations

Exclusion criteria

- Current use of tobacco, nicotine-containing products, or cannabis
- Presence of overhanging restorations, untreated carious lesions, or dental mineralization defects
- Systemic disease or known immunodeficiency (congenital or acquired)
- Current or recent use of immunosuppressive treatment
- Use of probiotics
- Use of treatments likely to affect oral health within the previous 3 months, including systemic antibiotics or chlorhexidine-containing mouthwashes.

Withdrawal criteria

- Participants may withdraw from the study at any time, without justification, by revoking their informed consent; withdrawal may occur before or after randomization and will not affect subsequent orthodontic care

Intervention and Comparator

Control Arm: Standard Care

Participants allocated to the standard care group will receive standard orthodontic preventive care routinely delivered at the participating centers. Standard care includes conventional oral hygiene instructions provided at appliance placement and during routine orthodontic follow-up visits, together with professional prophylactic cleaning according to usual clinical practice. To ensure consistency between centers, participating clinicians will follow harmonized preventive recommendations regarding oral hygiene instructions, preventive advice, and professional cleaning procedures.

Test Arm: Standard Care Plus Personalized Prevention

Participants allocated to the intervention group will receive, in addition to standard care, an intensive personalized prevention care at baseline and at each follow-up visit throughout the 12-month study period.

The intervention combines (1) individualized oral health education, (2) supervised toothbrushing sessions, (3) dietary counseling, (4) plaque disclosure and visualization, (5) repeated motivational reinforcement, and (6) standardized chairside prophylaxis using the Electro Medical Systems (EMS) protocol [36].

The personalized nature of the intervention is based on repeated individualized assessments performed longitudinally at each study visit. Preventive counseling and reinforcement are adapted according to each participant's oral hygiene practices, plaque accumulation patterns, dietary habits, compliance indicators, behavioral evolution over time, and participant-specific educational and motivational needs.

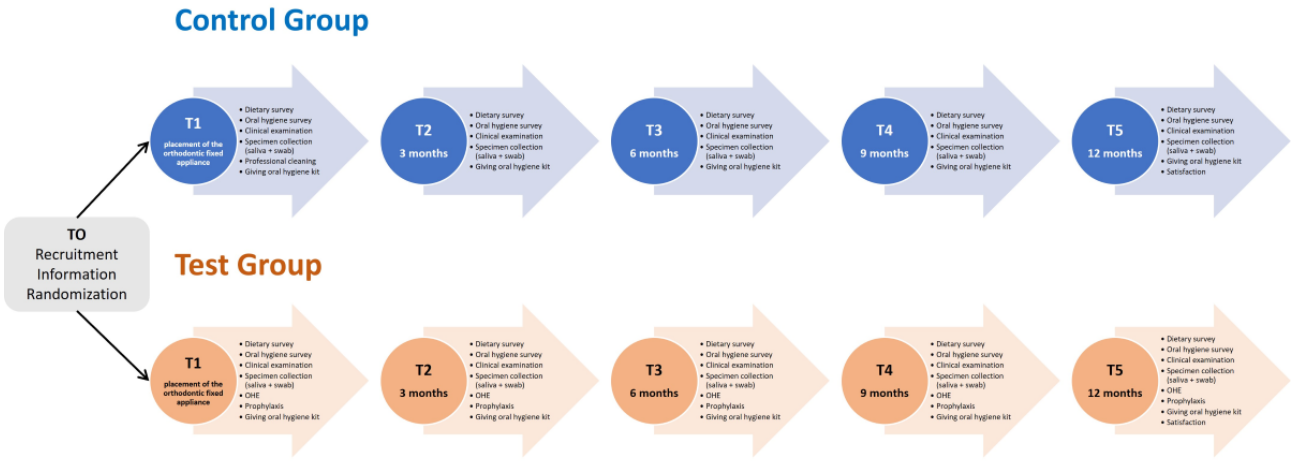
Thus, although the general preventive framework remains standardized across participants, the intensity, educational emphasis, behavioral reinforcement, and preventive support are individualized according to the participant's evolving clinical and behavioral profile during orthodontic treatment.

The intervention protocol was developed based on (1) existing preventive orthodontic practices, (2) current concepts in oral microbial ecology and dysbiosis prevention, and (3) the clinical expertise of the participating orthodontic and oral medicine teams.

As PREPERMIO is designed as an exploratory, biologically driven interventional study, no formal pilot trial was conducted before implementation. However, the preventive procedures included in the protocol are routinely used in clinical orthodontic practice and were harmonized across participating centers before study initiation. A schematic overview of the structure and components of the intervention and comparator arms, as well as the overall study timeline, is provided in Figure 1.

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Figure 1. PREPERMIO (Personalized Prevention During Fixed Orthodontic Treatment and Oral Microbial Homeostasis) study design and follow-up schedule. Randomized longitudinal study design of the PREPERMIO trial comparing a control group receiving standard preventive care and a test group receiving an individualized preventive intervention. After recruitment and randomization at T0 (information, inclusion, and allocation), participants are followed at T1 (baseline at appliance placement), T2 (3 months), T3 (6 months), T4 (9 months), and T5 (12 months). At each visit, clinical examination, oral hygiene assessment, dietary and hygiene questionnaires, saliva and biofilm sampling, and patient-reported outcomes are collected. OHE: oral hygiene education.



Outcomes

Primary Outcome and Assessment of Microbial Dysbiosis

The primary outcome of the PREPERMIO study is the longitudinal changes of predefined oral microbial ecological composition during the first year of fixed orthodontic treatment, comparing participants receiving standard preventive care alone with those receiving standard care plus personalized prevention. In the context of this study, dysbiosis is defined as a longitudinal ecological shift characterized by compositional and quantitative changes in the relative and absolute abundance of selected commensal and pathogenic microbial targets over time, particularly transitions toward increased representation of disease-associated bacterial complexes and reduction of health-associated microbial communities. Rather than focusing on a single isolated microorganism, the analytical framework is based on the time-resolved quantitative changes of predefined microbial ecological groups. Bacterial species will therefore be interpreted according to (1) their ecological role as pioneer, early, or late colonizers; (2) their association with health- or disease-associated bacterial Socransky complexes; and (3) their longitudinal quantitative evolution during orthodontic treatment.

The primary end point analysis will integrate both bacterial and viral targets to provide a broader assessment of oral ecological dynamics and host-microbe imbalance during fixed orthodontic treatment.

Secondary Outcomes

Secondary outcomes of the PREPERMIO study are to assess (1) host immune responses through quantification of salivary cytokine and chemokine levels, measured using the multiplex Meso Scale Discovery (MSD) platform; (2) clinical oral outcomes, including plaque accumulation assessed by Quigley-Hein plaque index after disclosure, bleeding on probing, gingival index, and white spot lesion count; (3) patient compliance, evaluated by hygiene device wear indices and toothpaste consumption by weight; and (4) patient satisfaction measured on a numerical scale 0-10 at end of the study.

Harms

The study is expected to be low-risk, with no medical procedures beyond standard orthodontic care. Potential harms are limited to minor discomfort, longer follow-up visits, or temporary irritation from oral hygiene procedures. Any issues reported by participants during the study will be documented by the study team and addressed promptly. All observations will be summarized in the final study report.

Sample Size

Formal sample size calculation was not feasible for this study because the main outcomes are exploratory microbiological and immunological end points. At present, no robust preliminary data or validated effect sizes are available for these outcomes in orthodontic patients, particularly in the context of personalized preventive interventions and repeated biological sampling. Moreover, microbial data are inherently high-dimensional, compositional, and multivariate, which limits the applicability of conventional power calculation methods typically applied to single, normally distributed variables.

The study therefore adopts a pragmatic sample size of 80 participants (40 per study arm), determined based on feasibility, recruitment capacity, and longitudinal follow-up constraints in a real-world orthodontic setting. This sample size is considered appropriate and informative for exploratory microbiome analyses, allowing the characterization of interindividual variability, the detection of biologically relevant trends over time, and meaningful comparisons between intervention and control groups. Importantly, this sample size will generate preliminary estimates of variability and group differences, which are currently unavailable, and will inform the design and sample size of future confirmatory trials.

Recruitment feasibility is supported by the active caseload of patients undergoing fixed orthodontic treatment in the

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participating centers, which is adequate to achieve the planned sample size within the defined inclusion period.

Randomization and Blinding

Randomization and Allocation Concealment

Participants will be randomly assigned in a 1:1 ratio to 1 of 2 study groups: standard preventive care alone or standard care combined with a personalized prevention strategy. Randomization will be performed centrally by the Clinical Research and Innovation Department (DRCI) of CHU Nice using an integrated REDCap (Vanderbilt University) randomization module. Randomization sequences will be generated using a block randomization method with random block sizes, created with nQuery software (version 9.0; Statistical Solutions Ltd).

Allocation will occur after written informed consent has been obtained and prior to initiation of orthodontic treatment. Investigators will access the secure electronic case report form (e-CRF) to register the participant and obtain the allocation assignment, ensuring allocation concealment until the moment of randomization.

Blinding

Due to the nature of the preventive intervention, the study will be conducted as an open-label trial, with neither participants nor clinicians blinded to group allocation. Clinical examinations will be performed using standardized protocols by trained examiners, acknowledging that, despite the open-label design, standardized procedures are used to ensure consistency across assessments. Patient-reported outcomes, including behavioral measures and satisfaction scores, will be collected using standardized questionnaires, which, although potentially influenced by participants' awareness of group allocation, are applied uniformly across both study arms. In addition, the laboratory personnel performing microbiological and immunological assays will be blinded to group allocation. To minimize assessment bias, biological samples will be pseudonymized using unique identification codes prior to laboratory analysis.

Patient Pathway and Study Procedures

Overview

Eligible patients requiring fixed orthodontic treatment, who meet all inclusion criteria and none of the exclusion criteria, will be invited to participate during an orthodontic assessment visit conducted either at the Institute of Oral Medicine of CHU Nice or at a private orthodontic practice in Dinan. The study will be explained by a trained practitioner or intern in dentofacial orthopedics, and written information will be provided. Participants and, where applicable, their legal representatives will be given sufficient time to consider participation before providing written informed consent. The duration of participation for each patient will be 12 months. The inclusion period is planned over 6 months. The overall study duration, including data analysis, is estimated at 24 months.

Study Schedule

The participant timeline for the PREPERMIO study is shown in Table 1.

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Table 1. SPIRIT (Standard Protocol Items: Recommendations for Interventional Trials) 2025 participant timeline for the PREPERMIO (Personalized Prevention During Fixed Orthodontic Treatment and Oral Microbial Homeostasis) study. Schedule of enrollment, interventions, and assessments for participants undergoing fixed orthodontic treatment. Screening includes eligibility assessment, provision of study information, and informed consent. Baseline (T0) corresponds to inclusion and appliance placement, with consent verification and randomization. Follow-up visits are scheduled at 3, 6, 9, and 12 months (±10 days). The personalized prevention intervention is delivered at each visit in the intervention group only. Clinical, microbiological, immunological, and compliance assessments are performed longitudinally as indicated.

Study procedures	–T1 to T0 (screening)	T0 (baseline)	T1 (3 months)	T2 (6 months)	T3 (9 months)	T4 (12 months/close-out)
Eligibility screen	✓					
Information provided/reflection period	✓					
Written informed consent obtained	✓					
Consent verification		✓				
Randomization (1:1)		✓				
Standard care (both groups)		✓	✓	✓	✓	✓
Personalized prevention (intervention group only)		✓	✓	✓	✓	✓
Demographics and medical history		✓				
Sociodemographic, dietary, and oral hygiene questionnaires		✓	✓	✓	✓	✓
Plaque index (Quigley-Hein)		✓	✓	✓	✓	✓
Gingival index + bleeding on probing)		✓	✓	✓	✓	✓
White spot lesion assessment		✓	✓	✓	✓	✓
Saliva sampling		✓	✓	✓	✓	✓
Biofilm sampling		✓	✓	✓	✓	✓
Compliance (hygiene device wear index)		✓	✓	✓	✓	✓
Compliance (toothpaste consumption by weight)		✓	✓	✓	✓	✓
Patient satisfaction (0-10 scale)						✓

T0: Screening (Orthodontic Assessment Visit)

Eligibility will be assessed, and participants will receive oral and written information about the study. The informed consent process will be initiated, allowing a reflection period prior to signature.

T1: Baseline (Inclusion and Start of Orthodontic Treatment)

Written informed consent will be confirmed prior to any study-specific procedure. Participants will then be randomized. Baseline data collection will include demographic characteristics (eg, age and sex), medical history, and completion of sociodemographic (eg, education level and employment status), dietary, and oral hygiene questionnaires. A baseline clinical oral examination will be performed. Biological samples will be collected, including saliva (by spitting) and dental biofilm (by swabbing at the tooth-gingiva junction). An oral hygiene kit will be provided to all participants. Participants allocated to the control group will receive standard professional cleaning, while participants allocated to the intervention group will receive a personalized prevention intervention.

T2: Follow-Up at 3 Months (±10 Days)

Participants will be assessed during a routine orthodontic follow-up visit. Follow-up questionnaires assessing changes in oral hygiene behaviors, dietary habits, and relevant health information since the previous visit will be completed. A clinical oral examination will be performed, and saliva and biofilm samples will be collected. The personalized prevention protocol will be applied in the intervention group.

T3: Follow-Up at 6 Months (±10 Days)

Procedures identical to the T2 visit will be performed, including clinical assessment and biological sampling. The personalized prevention protocol will be repeated in the intervention group.

T4: Follow-Up at 9 Months (±10 Days)

Procedures identical to the T2 and T3 visits will be performed. The personalized prevention protocol will be repeated in the intervention group.

T5: End-of-Study Visit at 12 Months (±10 Days)

Final questionnaires, clinical examination, and biological sampling will be conducted. Patient satisfaction will be assessed in both groups. The personalized prevention protocol will be applied in the intervention group. This visit marks the end of the participant's involvement in the study. Routine orthodontic care will then continue outside the study protocol.

Oral Biological Sampling and Sample Handling

Unstimulated whole saliva (2 mL) will be collected by passive drooling into sterile collection tubes. In addition, gingival biofilm samples will be obtained by swabbing the tooth-gingiva junction in the mandibular anterior region, between teeth 12 and 22, using a sterile swab. Participants will be instructed to

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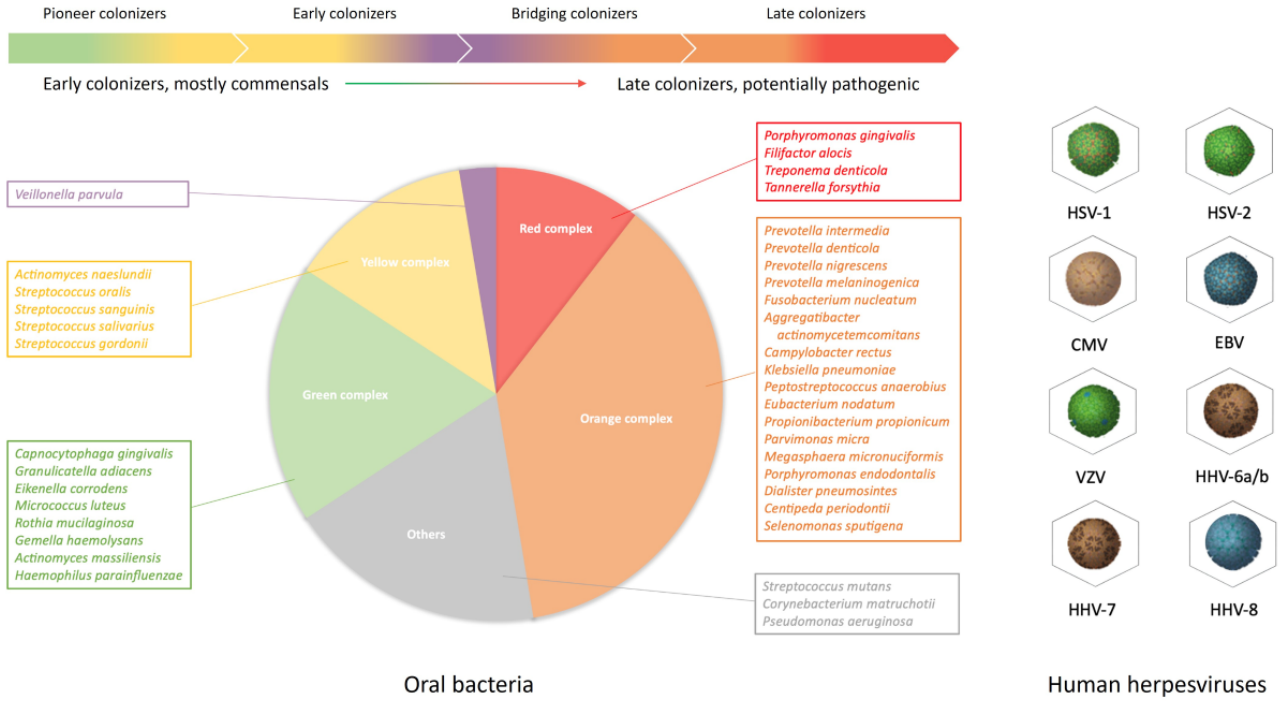
refrain from eating, drinking, toothbrushing, and smoking for at least 30 minutes prior to sampling [37].

Microbiological Analyses

Microbiological analyses will be performed using a targeted high-throughput microfluidic quantitative polymerase chain reaction (qPCR) platform based on the Biomark HD system (Standard Biotools). This targeted approach was selected instead of untargeted whole-metagenome sequencing because the study is hypothesis-driven and focused on predefined microbial groups with established clinical relevance in oral dysbiosis, rather than on exploratory microbiome profiling. It provides (1) high sensitivity for predefined clinically relevant microbial targets, including members of health-associated and disease-associated bacterial complexes (eg, Socransky complexes), as well as viral targets; (2) standardized longitudinal quantification across repeated measurements, which is essential for intergroup and intraindividual comparisons in a randomized multicenter design; (3) reduced analytical complexity for longitudinal ecological monitoring of predefined microbial groups; and (4) lower cost, higher throughput, and greater scalability for translational and clinically applicable research settings.

The targeted analytical panel includes a curated selection of commensal and pathogenic oral bacterial species representative of distinct oral health states, as well as all 9 viruses from the human herpesvirus (HHV) family. Target selection is based on previously established protocols and expertise developed within the MICORALIS Laboratory (Figure 2) [37-40]. Although this targeted approach does not capture the full taxonomic breadth accessible through untargeted sequencing technologies, it was considered an appropriate methodological choice for a hypothesis-driven longitudinal interventional study, ensuring analytical robustness, clinical interpretability, and feasibility while maintaining alignment with the predefined biological objectives.

Figure 2. Targeted oral microbial panel analyzed in the PREPERMIO (Personalized Prevention During Fixed Orthodontic Treatment and Oral Microbial Homeostasis) protocol. Schematic representation of the bacterial and viral species included in the study. The bacterial panel comprises both commensal and pathogenic oral species, classified by Socransky microbial complexes (green, yellow, purple, orange, and red) and by their role as pioneer, early, or late colonizers. This dual classification enables monitoring of shifts in microbial balance over time, capturing transitions from commensal-dominated to pathogenic-dominated communities, indicative of dysbiosis. All 9 HHVs are also included to assess viral signatures that may contribute to, or reflect, changes in the oral microbial ecosystem of orthodontic patients [37]. CMV: cytomegalovirus; EBV: Epstein-Barr virus; HSV: herpes simplex virus; VZV: varicella-zoster virus.



All experiments will be conducted using 96.96 integrated fluidic circuit plates, following the instructions of reagent kits (Standard BioTools). A preamplification step will be performed to increase the quantity of target complementary DNA (cDNA) from low-abundance bacterial and viral sequences, ensuring sufficient template for sensitive and accurate downstream qPCR detection, according to the protocol "*Preamplification of cDNA for Gene Expression with Delta Gene Assays*." Following preamplification, samples will be treated with Exonuclease I and diluted 1:20 to remove residual single-stranded primers, preventing interference with downstream amplification. Diluted samples will then be processed according to the protocol "*Gene Expression with the 96.96* IFC *using Delta Gene Assays on Preamplified Samples*," using the AX SsoFast EvaGreen Supermix with low ROX (Bio-Rad) to enable rapid and efficient fluorescent qPCR amplification of the target DNA. The reaction workflow further includes an initial uracil-N-glycosylase treatment at 50 °C for 2 minutes to eliminate potential carryover contamination, followed by a hot-start activation phase at 95 °C for 10 minutes. Polymerase chain reaction (PCR) amplification will then proceed for 40 cycles, consisting of denaturation at 95 °C for 15 seconds and annealing/extension

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at 60 °C for 60 seconds, with fluorescence data acquisition at each annealing step.

Each sample will be analyzed in duplicate and normalized to 10 ng/µL for saliva samples and 1 ng/µL for gingival swab samples. A total volume of 1.25 µL of template DNA will be used per reaction, corresponding to 12.5 ng of DNA for saliva samples and 1.25 ng for swab samples. Data analysis will be performed using the Real-Time PCR Analysis Software (Fluidigm) with automatically defined thresholds. Samples presenting quantification cycle values ≥25 will be considered negative for the corresponding target.

Final quantification results will be normalized using standard curves generated from theoretical copy numbers calculated for each microorganism based on genomic DNA concentration (ng/µL) and genome size. Tenfold serial dilutions ranging from 10^8 to 1 copy/µL were prepared and analyzed by qPCR. Standard curves were constructed by plotting cycle threshold (Ct) values against the $\log_{10}$-transformed copy numbers. Absolute quantification will be determined from these calibration curves and expressed as copy number per µg of DNA for each microorganism in each sample. Copy numbers will be calculated using the equation: $\log_{10}(\text{copies}) = (Ct - b)/a$, where *a* represents the slope and *b* the intercept of the linear regression curve. Absolute values will be subsequently normalized to µg of input DNA.

For the longitudinal analyses, the main variables will be the changes over time in (1) absolute copy numbers of each microbial target, (2) relative abundance of health-associated and disease-associated microorganisms, and (3) the balance between predefined microbial ecological groups, including Socransky complexes. Analyses will compare both within-subject changes across follow-up visits and differences between the standard care and personalized prevention groups. Therefore, the primary outcome is based on the direction and magnitude of longitudinal microbial changes during orthodontic treatment rather than the simple presence or absence of microorganisms.

Assessment of Microbial Shifts and Dysbiosis

To explore shifts in microbial composition associated with fixed orthodontic treatment, we will quantify both absolute and relative abundances of bacterial targets over time, considering the ecological succession of oral biofilms. Bacterial species will be grouped broadly according to their colonization patterns: pioneer, early, and late colonizers, while referencing health- and disease-associated complexes (eg, Socransky green, yellow, purple, orange, and red complexes) to provide context for pathogenic potential (Figure 2). Longitudinal changes in these groups will be tracked to identify progressive imbalances that may arise from inadequate plaque removal or the presence of retentive orthodontic surfaces. Comparative analyses between the standard care and personalized prevention groups will allow assessment of how targeted interventions influence biofilm composition and the transition from commensal-dominated to pathogenic-dominated communities. Viral targets will also be integrated to provide a holistic view of oral ecological dynamics.

Immunological Analyses

For immunological analyses, cytokine levels will be quantified using the MSD MULTI-ARRAY technology, an electrochemiluminescence-based multiplex platform that enables sensitive detection of inflammatory markers associated with microbial changes. This patterned array system offers high sensitivity, a broad dynamic range, and multiplexing capability. The S-PLEX kit will be used, allowing the simultaneous quantification of 9 cytokines per sample, with detection limits in the femtogram per milliliter (fg/mL) range.

Assays will be performed following the manufacturer's protocols. Briefly, precoated patterned array plates are first blocked to minimize nonspecific binding, after which saliva samples and calibrators are added. Detection antibodies are then applied, followed by a signal-enhancement step that generates an electrochemiluminescent signal proportional to cytokine concentration. Plates are washed between each step to reduce background noise. Electrochemiluminescent signals are measured using an MSD reader (MESO QuickPlex SQ 120MM). Cytokine concentrations are calculated from analyte-specific standard curves. All samples will be analyzed in duplicate, and internal quality controls will be included on each plate to ensure assay reliability and reproducibility. Where applicable, results will be normalized to total protein content [37].

Use and Disposal of Biological Samples

No biological collection will be constituted for this study. All biological samples will be used exclusively for the analyses described in this protocol and will not be stored for future research. All remaining samples will be destroyed after completion of laboratory analyses, in accordance with French regulatory requirements.

Data Collection, Management, and Analysis

Data Collection Methods, Data Management, and Participant Retention and Follow-Up

As detailed in the relevant clinical, biological, and laboratory sections of this protocol, study data will be collected through participant-reported questionnaires, standardized clinical examinations, and biological sample analyses at predefined study visits. This section focuses on data recording, management, and analysis procedures.

Study data will be recorded using a dedicated e-CRF hosted in REDCap, a secure web-based data capture platform maintained by the DRCI of CHU Nice. The principal investigator (LL) oversees the overall conduct of the study. The e-CRF will capture all clinical, microbiological, immunological, and compliance-related data at each scheduled study visit, using standardized variable definitions and predefined response formats. The e-CRF will be based on a finalized paper case report form and configured to ensure standardized and secure data collection.

Investigators will enter study data directly into the e-CRF, with support from the study clinical research associate as needed. Access to the database will be role-based, with predefined user permissions to ensure data security and participant confidentiality. Data quality will be ensured through systematic

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validation and consistency checks performed by the DRCI data manager. Queries will be generated as required to resolve inconsistencies or missing data. All modifications to the database will be documented through an audit trail.

At the end of the data cleaning process, the database will be locked following validation by the principal investigator, the data manager, and the DRCI physician. The locked database, together with the data management report, will then be transferred to the statistician for statistical analysis. No further changes to the data will be permitted after the database lock.

To promote participant retention and complete follow-up, study visits will be coordinated with routine orthodontic appointments whenever possible. Participants will receive visit reminders and follow-up contacts in case of missed appointments. In the event of premature discontinuation or deviation from the intervention protocol, outcome data already collected up to the time of withdrawal will be retained and included in the analysis whenever permissible.

Statistical Analysis

Statistical analyses will be performed by a biostatistician from the DRCI of CHU Nice using SAS Enterprise Guide software (version 7.1; SAS Institute Inc). All analyses will be conducted according to a prespecified statistical analysis plan. All statistical tests will be 2-sided and conducted at a significance level of 5%. Assumptions underlying each statistical test will be checked prior to analysis, and alternative methods will be applied when these assumptions are not met.

Analysis Populations

The primary analysis will be conducted in the modified intention-to-treat population, defined as all randomized participants who have completed at least 1 postbaseline assessment (≥3-month follow-up visit, T2). Participants will be analyzed according to their initial group allocation regardless of adherence to the assigned intervention.

A complementary per-protocol analysis will also be performed among participants considered compliant with the preventive strategy (eg, toothbrushing at least twice daily and meeting predefined toothpaste consumption criteria). Participants with major protocol deviations or incomplete follow-up will be excluded. Results from the per-protocol analysis will be considered supportive and exploratory, aimed at evaluating the effect of the intervention under optimal adherence conditions, and will not replace the primary modified intention-to-treat analysis.

Descriptive Analyses

Baseline characteristics and longitudinal follow-up data will be summarized using descriptive statistics. Categorical variables will be reported as counts (n) and percentages (%) with 95% CIs, while quantitative variables will be summarized using means and SDs for normally distributed data, or medians and IQRs for nonnormally distributed data.

Baseline characteristics will be summarized separately for each study group using descriptive statistics. No statistical tests will be performed to compare groups at baseline, in line with CONSORT (Consolidated Standards of Reporting Trials) guidelines, since randomization is expected to balance the groups. A flow diagram will describe participant inclusion, randomization, follow-up, and analysis populations. Characteristics of participants who discontinue the study or are lost to follow-up will also be reported.

Analysis of the Primary End Point

The primary end point concerns longitudinal ecological shifts in predefined microbial targets and microbial ecological groups during fixed orthodontic treatment. Primary longitudinal microbial outcomes will be analyzed using linear mixed effects models, which are considered appropriate for repeated longitudinal measurements characterized by within-subject correlations, interindividual variability, and potential missing data.

Models will include (1) study group, (2) time, and (3) group-by-time interaction as fixed effects, with participant-level random effects to account for individual differences at baseline and in response over time (ie, repeated measurements within the same participant).

The primary analytical framework will focus on within-individual longitudinal changes and between-group differences in temporal trajectories of predefined microbial variables rather than isolated cross-sectional comparisons at individual time points.

The primary outcome related to dysbiosis will therefore be defined as longitudinal changes across multiple predefined microbial targets, reflecting coordinated increases or decreases in health-associated and disease-associated microbial groups, including viral targets. These changes will be evaluated at the level of predefined microbial groups rather than single species alone.

Analysis of Secondary End Points

Longitudinal changes in salivary cytokine and chemokine levels will be analyzed using mixed effects modeling approaches similar to those applied to the primary end point.

Clinical outcomes and compliance measures will be compared between groups using appropriate parametric or nonparametric statistical methods depending on variable distribution and outcome type. Patient satisfaction will be compared between groups at the end of follow-up (T5) using chi-square or Fisher exact tests, as appropriate.

Multiplicity Adjustment

Multiplicity adjustment procedures, including false discovery rate correction where appropriate, will be applied to control for multiple testing within microbial and inflammatory end point analyses involving multiple bacterial, viral, and immune markers.

Missing Data

Mixed effects modeling inherently allows robust handling of incomplete repeated-measures data under missing-at-random assumptions. Sensitivity analyses using additional imputation approaches may also be considered where appropriate.

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Complementary Exploratory Analyses

Repeated-measures ANOVA may be performed as a complementary exploratory approach when statistical assumptions are adequately met.

Ethical Considerations

Overview

This trial will be conducted in accordance with the Declaration of Helsinki and applicable French regulations governing research involving human participants, including the Jardé law framework. The study protocol, participant information sheet, and informed consent forms have been approved by the French Committee for the Protection of Persons (CPP 2024-A00646-41; December 6, 2024), and the French National Agency for the Safety of Medicines and Health Products (ANSM) has been notified (December 20, 2024).

All participants will be fully informed about the study objectives, procedures, potential benefits, and constraints. Written informed consent will be obtained prior to any study-specific procedure. For participants aged <18 years, consent will be obtained from the legal guardians in accordance with French legislation. Participants will be informed of their right to decline participation or withdraw from the study at any time without justification and without any impact on their ongoing orthodontic care.

Data Confidentiality

All medical and nonmedical staff involved in the study will be bound by professional and medical confidentiality. Participant data will be collected and stored in a secure electronic database with restricted access. Data will be pseudonymized using unique study identifiers, and all analyses will be conducted on coded datasets in accordance with data protection regulations.

Risk-Benefit Assessment

The anticipated risk-benefit balance of the study is considered favorable. No additional medical risks are expected beyond standard orthodontic care. The main constraint for participants relates to the longer duration of some follow-up visits, particularly in the intervention group, due to the delivery of personalized preventive procedures.

Potential individual benefits include enhanced oral hygiene support for participants in the intervention group. At a collective level, the study may contribute to improved understanding of oral microbial shifts and dysbiosis during fixed orthodontic treatment and inform the development of more effective preventive strategies.

Dissemination of Results

Study results will be disseminated through publication in peer-reviewed scientific journals and presentation at national and international conferences. Participants will be informed that aggregated study findings may be made available upon request.

Results

The PREPERMIO study received approval from the French Committee for the Protection of Persons (CPP 2024-A00646-41) on December 6, 2024. The study was notified to the ANSM on December 20, 2024. The trial was prospectively registered on ClinicalTrials.gov (NCT06752902) prior to participant recruitment.

Participant recruitment had not yet started, confirming the prospective and prerecruitment status of the study (prerecruitement stage). Study preparation phases include finalization of study documents, e-CRF configuration, personnel calibration, and laboratory workflow standardization prior to recruitment initiation.

Participant enrollment is projected to occur between March and September 2026 across the 2 participating centers. Each participant will be followed for 12 months after appliance placement, with scheduled assessments performed at baseline and at 3, 6, 9, and 12 months.

Biological sample processing, microbiological and immunological analyses, database cleaning, and database lock will be conducted progressively following completion of follow-up. Final statistical analyses and dissemination of study findings are anticipated in early 2028. The overall study duration is estimated at approximately 24 months.

The participant timeline and study phases are presented in the SPIRIT (Standard Protocol Items: Recommendations for Interventional Trials) schedule table and the complementary study flow diagram (Figure 1).

Discussion

Principal Contribution and Novelty

The adhesion and maturation of dental biofilm in the presence of orthodontic appliances remain a major clinical concern, contributing to enamel demineralization, white spot lesions, and periodontal diseases. Areas that are difficult to access for cleaning, such as interdental spaces, occlusal grooves, and orthodontic appliances, favor increased biofilm retention and accumulation [41]. Oral diseases such as periodontitis and dental caries are driven by microbial community imbalances, in which the transition from commensal-dominated to pathogenic-dominated communities (ie, dysbiosis) plays a major etiological role. Consequently, characterizing changes in the composition and abundance of health- and disease-associated oral microorganism is essential for the development of new therapeutic and preventive strategies [21,42].

PREPERMIO is among the first randomized interventional studies to evaluate a personalized preventive strategy during fixed orthodontic treatment using biologically driven end points. By integrating microbiological, virological, immunological, clinical, and behavioral dimensions over a 12-month period, this protocol aims to provide a comprehensive assessment of oral ecological dynamics. Unlike most existing orthodontic prevention studies, which focus on clinical outcomes or behavioral interventions over short periods (Table 2) [29-31,43],

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PREPERMIO integrates clinical indices and behavioral measures while placing microbial homeostasis and dysbiosis at the center of the primary end point, with the addition of HHVs and host inflammatory markers enhancing biological relevance. This integrative "host-microbe behavior" framework may provide novel insights into viral-bacterial-host interactions during fixed orthodontic treatment.

Table 2. Comparative overview of PREPERMIO (Personalized Prevention During Fixed Orthodontic Treatment and Oral Microbial Homeostasis) and previously published orthodontic prevention protocols. Comparison of study designs, interventions, outcomes, and biological approaches across orthodontic prevention protocols.

Study/protocol	Study design and population	Intervention	Primary outcome	Biological approach	Follow-up
PREPERMIO (present protocol)	Multicenter RCT[a]; participants aged 12-20 years; fixed orthodontic appliances	Repeated personalized prevention + standard care vs standard care alone	Microbial dysbiosis (bacterial and viral targets)	Targeted high-throughput microfluidic qPCR[b] (bacterial and HHV[c] targets) + salivary cytokine profiling	12 months (0, 3, 6, 9, and 12)
Scheerman et al [29]	RCT; orthodontic adolescents	Oral hygiene mobile app	White spot lesions; oral health behavior	No central microbiological end point	12 months
Sharif et al [30]	RCT; feasibility study; orthodontic adolescents	Personalized digital intervention	Adherence and acceptability	No biological assessment	Short- to midterm
Chen et al [43]	RCT; orthodontic/periodontal population	Periodontal intervention	Periodontal clinical indices	Microbiological assessment as secondary outcome	Long-term
Koopman et al [31]	RCT; orthodontic patients	Oral hygiene control strategy	Changes in microbial composition	Microbiome analysis (16S rRNA[d] sequencing)	Short-term

[a]RCT: randomized controlled trial.

[b]qPCR: quantitative polymerase chain reaction.

[c]HHV: human herpesvirus.

[d]rRNA: ribosomal RNA.

Bacterial Composition and Ecological Shifts

Supragingival biofilm is readily detected during clinical examination using a periodontal probe or disclosing agents. It is composed predominantly of Gram-positive aerobic and facultative anaerobic bacteria, including health-associated streptococci such as *Streptococcus sanguinis*, *S oralis*, *S mitis*, *S salivarius*, and *S gordonii* [44,45]. These species are among the first colonizers of tooth and orthodontic appliance surfaces, adhering directly to the acquired salivary pellicle and contributing to biofilm initiation and structural stability [46]. As biofilm biomass increases and local ecological conditions change, particularly with increased carbohydrate availability and a sustained decrease in pH, other streptococcal species with higher acidogenic and aciduric potential, such as cariogenic *S mutans*, may become selectively enriched [47]. This ecological shift favors the progression from a commensal-dominated biofilm toward a more cariogenic and inflammatory phenotype. With further biofilm maturation, sequential recruitment of secondary and late colonizers occurs, facilitating increased microbial complexity and the emergence of bacterial communities with enhanced pathogenic and proinflammatory potential.

In contrast, subgingival biofilm is much more difficult to assess clinically. It colonizes the gingival sulcus and periodontal pockets, which are oxygen-deprived environments characterized by reduced redox potential and altered pH [48]. These conditions favor the growth of obligate anaerobic and more virulent bacteria that are primarily associated with periodontal inflammation and periodontal diseases. The transition from a predominantly supragingival, commensal-dominated biofilm to a subgingival, anaerobe-enriched biofilm reflects a critical ecological shift toward dysbiosis.

Differences in bacterial composition between subgingival biofilm samples collected from periodontally healthy individuals and patients with periodontitis have been extensively studied, leading to the classification of Socransky's bacterial complexes (Figure 2) [49]. Subgingival plaque samples from healthy individuals mainly contain *Actinomyces* species and, to a lesser extent, *Streptococcus* species (yellow complex) and *Veillonella* species (purple complex). Fewer than 4% of the total identified bacteria belong to the orange complex (*Fusobacterium nucleatum*, *Prevotella* species, and *Campylobacter rectus*) or the red complex (*Porphyromonas gingivalis*, *Tannerella forsythia*, *Treponema denticola*, and *Filifactor alocis*), both of which are strongly associated with periodontal disease [50]. An increase in these pathogenic complexes has also been observed in mature biofilms cultured from samples obtained from both healthy individuals and patients with periodontitis, suggesting that dysbiosis reflects not merely the presence of specific pathogens but rather a shift in relative abundance, spatial organization, and ecological dominance within the microbial community. By integrating bacterial classification according to both colonization sequence (early or late colonizers) and Socransky complexes, the PREPERMIO protocol is specifically designed to capture these dynamic transitions from commensal-dominated to pathogenic-dominated biofilms,

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thereby providing a biologically grounded framework for monitoring dysbiosis during fixed orthodontic treatment.

Comparison With Published Orthodontic Prevention Protocols

In orthodontics, prevention trials and protocols most frequently evaluate behavioral interventions (eg, mobile apps to improve hygiene adherence) or preventive strategies aimed at clinical end points such as caries or white spot lesions (Table 2). For example, protocols in orthodontic adolescents have assessed digital or behaviorally oriented interventions to improve oral hygiene behavior and caries prevention. These studies are essential but often prioritize behavioral adherence and observable clinical effects rather than providing a detailed characterization of the underlying biological processes or microbial signatures associated with dysbiosis.

In contrast, the PREPERMIO protocol places microbial homeostasis at the core of the primary end point and extends biological profiling beyond bacteria to include viral targets, namely HHVs, an emerging dimension of periodontal and mucosal inflammatory ecology [39,51,52]. By integrating bacterial, viral, and host inflammatory parameters within a longitudinal design, PREPERMIO moves beyond outcome-based prevention toward a mechanistic understanding of ecological shifts during fixed orthodontic treatment. This integrative approach has the potential to provide novel insights into viral-bacterial-host interactions and their contribution to dysbiosis in orthodontic patients.

Strength

The major strengths of this study include the following. First, integrative longitudinal design: the study combines targeted high-throughput quantification of selected bacterial and viral targets in both saliva and dental biofilm with assessment of the host inflammatory response through cytokine and chemokine profiling, alongside clinical outcomes and objective measures of patient compliance. This comprehensive “host-microbe behavior” framework is intended to enhance mechanistic understanding of microbiota dynamics during orthodontic treatment and to identify biologically informed risk profiles that may support the development of personalized preventive approaches. Second, the multicenter randomized design, incorporating both public hospital and private practice settings, strengthens external validity and increases the generalizability of the findings. Third, repeated longitudinal assessments: repeated assessments over a 12-month period allow evaluation of dynamic ecological and inflammatory changes rather than cross-sectional comparisons. Fourth, objective compliance measures: the inclusion of objective compliance measures, such as device wear and toothpaste consumption, reduces reliance on self-reported data and may limit reporting bias. Fifth, scalable microbiological profiling: the use of high-throughput targeted quantitative PCR represents a potentially translatable and scalable approach for clinical microbiological profiling in orthodontic prevention.

Limitations

Several limitations of the PREPERMIO should be acknowledged. (1) Open-label design: due to the nature of the intervention, blinding of participants and clinicians is not feasible. However, the primary outcomes are based on objective laboratory measurements, and the use of coded samples before analysis is expected to reduce performance and detection bias. (2) Sample size estimation: the lack of comparable prior data prevented formal sample size estimation. This study should therefore be viewed as a pragmatic interventional trial designed to generate effect size estimates and inform future confirmatory studies. (3) Targeted microbial panel: from a laboratory perspective, although the targeted microbial panel enables scalable and clinically feasible quantification, it does not capture the full taxonomic breadth achievable with metagenomic sequencing. Nevertheless, targeted panels offer a pragmatic balance between biological relevance and implementation in clinical settings. (4) Generalizability: the inclusion of young, otherwise healthy participants (12-20 years) aligns with a primary prevention framework but may limit generalizability to older or medically compromised populations. (5) Missing data handling: the use of last observation carried forward for missing data, while conservative, may introduce bias. Complementary sensitivity analyses using mixed effects models with maximum likelihood estimation could be considered in the final report.

Perspectives and Potential Impact

The PREPERMIO protocol addresses a clinically important gap in orthodontic care by targeting the ecological shifts of the oral microbiota during fixed appliance treatment. By integrating bacterial, viral, and host inflammatory markers in a longitudinal, personalized prevention framework, the study moves beyond conventional outcome-based protocols toward a mechanistic understanding of oral microbial community transitions and ecological imbalance.

If the personalized preventive strategy demonstrates a favorable impact on microbial homeostasis and inflammatory biomarkers, the findings may support a shift from standardized preventive protocols toward stratified or personalized preventive care in orthodontics, allowing clinicians to identify individuals at higher biological risk for plaque accumulation, gingival inflammation, or caries and to tailor preventive strategies accordingly. The inclusion of HHVs as viral biomarkers further extends the understanding of viral-bacterial-host interactions in oral ecology, potentially informing interventions that address both microbial and host inflammatory drivers of disease.

Beyond orthodontics, the insights gained from PREPERMIO could inform broader oral health strategies by establishing scalable, clinically translatable frameworks for monitoring microbial and immunological homeostasis, ultimately improving preventive care and reducing the long-term burden of oral diseases.

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Data Availability

No datasets are generated or analyzed at the protocol stage. Data generated during the study will be available from the corresponding author upon reasonable request and in accordance with applicable regulations.

Authors' Contributions

MD, MO, LT, MFB, and LL contributed to the conceptualization and design of the study. MD, MO, LT, and MFB contributed to the development of the intervention and data collection procedures. LL is the principal investigator and guarantor of the study. OS contributes as an investigator in the private practice setting. All authors contributed to drafting and critically revising the manuscript and approved the final version.

Conflicts of Interest

None declared.

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Abbreviations

ANSM: French National Agency for the Safety of Medicines and Health Products
cDNA: complementary DNA
CHU Nice: Nice University Hospital
CONSORT: Consolidated Standards of Reporting Trials
CPP: Committee for the Protection of Persons
Ct: cycle threshold
DRCI: Clinical Research and Innovation Department
e-CRF: electronic case report form
EMS: Electro Medical Systems

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HHV: human herpesvirus
MSD: Meso Scale Discovery
PCR: polymerase chain reaction
PREPERMIO: Personalized Prevention During Fixed Orthodontic Treatment and Oral Microbial Homeostasis
qPCR: quantitative polymerase chain reaction
SPIRIT: Standard Protocol Items: Recommendations for Interventional Trials

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