affecting the oral cavity (including antibiotics) taken within the last 3 months; no teeth with active dental caries and/or missing teeth due to caries; and absence of periodontal disease. The patients' orthodontic treatment plan did not include tooth extractions or other mechanics requiring the use of bands on molars. Ethical Board approval was obtained from both institutes prior to study initiation (S249/31.7.2014 and P076/AD6271/30.3.2017) and informed consent was obtained from all patients or their guardians.

The patients were assigned to one of the following two groups: (i) treatment with self-ligating fixed appliances and nickel-titanium (NiTi) archwires in both arches (In-Ovation R brackets and Sentalloy Wire 0.014 in.—both from GAC International, Central Islip, New York, USA) or (ii) treatment with passive aligners constructed from clear transparent polyethylenterephthalat-glycol copolyester (PET-G) thermoplastic sheets (0.75 mm in thickness, Duran®+, Scheu Dental, Iserlohn) for 1 month. Aligners were used for 1 month experimentally and the patients were afterwards treated with fixed appliances. The thermoplastic PET-G sheets were pressed over a dental stone model according to the manufacturer's instructions, employing the Essix® Vacuum Thermoforming Machine (Dentsply Raintree Essix).

Sample size calculation

Sample size calculation was based on a previous study [14] that reported mean log-*S. mutans* counts per milliliter saliva following appliance bonding of 4.57 with a standard deviation (SD) of 1.17. Assuming a 30% reduction in the *S. mutans* counts for aligners and a common SD, 13 patients per group would be needed to achieve power of 80% at alpha of 5% with a Student's *t* test for independent samples. This was rounded up to 15 patients per group to account for data losses, to a total sample of 30 patients overall.

Clinical protocol

Each patient received professional oral care and standardized hygiene instructions 3 weeks before the beginning of orthodontic treatment/insertion of the thermoplastic appliances using a typodont model, with specific attention to fixed appliance care. Additional instructions were given to brush the thermoplastic appliances once daily. The bonding procedure was performed with the direct technique using Transbond-XT (3M Unitek, Monrovia, Calif). Patients were instructed to wear the thermoplastic appliances full time, except when eating, drinking, or brushing their teeth. These appliances were replaced after 2 weeks with a new set.

All patients were asked to refrain from eating, drinking, and brushing 2 h prior to all clinical examination and saliva collection. These procedures were performed in a dental chair between 09:00 and 12:00 a.m. For each participant, the following clinical variables were assessed: the simplified plaque index (s-PlI), where the percentage of surfaces with plaque is recorded (taking into consideration four surfaces per tooth for all erupted teeth); the simplified gingival index (s-GI), where the presence or absence of gingival bleeding after gentle probing of the gingival margin is recorded at six sites around all fully erupted teeth; and the decayed, missing, and filled teeth (DMFT) index for the prevalence of caries. The indices were recorded after each saliva sample collection at each visit without the use of a plaque disclosing agent. DMFT index was recorded using criteria of the World Health Organization for permanent dentition [20]. All the clinical measurements within each one of the two experimental groups were performed by the same calibrated investigator (IS and AP).

Sample collection and examination

Whole stimulated saliva was collected from each patient at three time points: (i) at baseline (T0), before bonding and initiation of orthodontic therapy, or before insertion of the thermoplastic aligners; (ii) after 2 weeks (T1); and after 1 month (T2). At all three time points, each patient chewed a paraffin gum for 5 min and spitted into plastic cups, while flow rate was calculated as milliliter per minute. From each patient, 1 ml of saliva was used to calculate the buffer capacity using a commercial buffer capacity test (CRT-buffer; Ivoclar, Vivadent, Liechtenstein). Collection of saliva samples was performed before any oral examination or manipulation so as not to disrupt the oral microbiota.

For the quantification of salivary cariogenic species (*S. sanguinis*, *L. acidophilus*, and *S. mutans*), 300 μl of stimulated saliva was transferred to sterile Eppendorf plastic vials adding 300 μl Tris EDTA buffer (TE buffer, 10 mM Tris-HCL, 1 mM EDTA, pH 7.6) and 300 μl 1 M NaOH solution. Samples were prepared in triplicate and kept frozen at − 80 °C until transported to the Laboratory of Microbiology, School of Dentistry, University of Athens, where they were used for the detection and quantification of salivary bacteria with quantitative polymerase chain reaction (qPCR).

Statistical analysis

The primary outcome of this study was the salivary counts of *S. mutans*, while the secondary outcomes were the salivary counts of *L. acidophilus* and the salivary counts of *S. sanguinis*. The periodontal parameters (s-PII and s-GI) of all patients were also measured to assess their influence on the salivary levels of the bacteria. Data normality was assessed with graphs and tested formally with the Shapiro-Wilk test. In order to