Salivary levels of cariogenic bacterial species during orthodontic treatment with thermoplastic aligners or fixed appliances: a prospective cohort study

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DOI: https://doi.org/10.1186/s40510-018-0230-4

Salivary levels of cariogenic bacterial species during orthodontic treatment with conventional brackets or thermoplastic aligners.

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Prog Orthod. 2018 Aug 1;19(1):25. doi: 10.1186/s40510-018-0230-4.

Abstract

Background: Fixed orthodontic appliances may be considered a risk factor to the integrity of enamel due to plaque accumulation and their colonization by oral microbes. However the demand for orthodontic treatment with esthetic clear thermoplastic aligners orthodontic continues to grow significantly. These removable appliances may behave differently regarding bacterial colonization and biofilm formation. The aim of this prospective comparative cohort study was to assess the salivary prevalence of the cariogenic bacteria S. mutans, L. acidophilus and S. sanguinis among adolescents treated orthodontically with thermoplastic aligners or fixed appliances.

Methods: 30 patients were assigned to one of the following 2 groups: (i) treatment with conventional fixed appliances and Nickel-Titanium archwires in both arches or (ii) treatment with passive aligners constructed from clear transparent polyethylenterephthalat - glycol copolyester (PET-G) thermoplastic sheets. Whole stimulated saliva was collected from each patient at three time points: at baseline (before bonding and initiation of orthodontic therapy or before insertion of the thermoplastic aligners), after 2 weeks and after 1 month. For each participant, the following clinical variables were assessed: the simplified plaque index, the simplified gingival index and the decayed, missing, and filled teeth (DMFT) index for the prevalence of caries. qPCR was used for the detection and quantification of salivary bacteria.

Results: Aligner patients had lower s-GI and s-PII scores compared to bracket patients throughout treatment. s-PII variation through time differed between aligner patients (where it tended to decrease through time) and bracket patients (where it tended to increase through time). S. sanguinis counts showed a tendency to reduce through time among the aligner patients, while they tended to increase through time

among the bracket patients. S. mutans counts were similar between the two groups at all times. Almost no L. acidophilus were identified in the collected saliva samples.

Conclusion: Lower salivary levels of S. sanguinis were found in adolescent patients treated for one month with thermoplastic aligners compared to those treated with conventional fixed appliances. On the other hand, no differences could be found regarding L. acidophilus and S. mutans.

Keywords: Aligner, Fixed appliances, S. mutans, S. sanguinis, L. acidophilus

Background

Although fixed appliances have revolutionized contemporary orthodontic treatment, they can at the same time be considered a risk factor to the integrity of enamel due to plaque accumulation and their colonization by oral microbes (Zachrisson, 1974). The placement of fixed orthodontic appliances complicates the use of standard oral hygiene procedures and causes alterations in the oral microflora by reducing pH, as well as by increasing plaque accumulation and the affinity of bacteria to metallic surfaces due to electrostatic reactions (Ahn et al., 2007). The insertion of fixed appliances creates new retentive areas that favor the local growth of streptococci, which in turn increase the levels of these organisms in saliva and around orthodontic appliances (Øgaard et al., 1988). This indicates that orthodontic brackets could act as a potential risk factor for enamel demineralization (Mitchell, 1992), which has been observed even only one month post-insertion (Gorelick et al., 1982).

Streptococcus mutans (S. mutans) and *Streptococcus sobrinus* (S. sobrinus) have been identified as the main contributors in the pathogenesis of dental caries and their presence contributes to the risk for enamel demineralization (Babaahmady et al., 2007). Increased levels of *S. mutans* and *Lactobacillus* species have also been reported to be detected in the oral cavity after bonding orthodontic attachments and some studies have reported that there is a positive correlation between dental caries and the degree of infection with these bacterial species (Lundstrom & Krasse, 1987a,b).

The levels of adhesion of these bacteria to fixed appliances might influence the formation of pathogenic plaque and enamel demineralization during orthodontic treatment (Babaahmady et al., 2007). The levels of adhesion of cariogenic streptococci to various orthodontic raw materials were evaluated in order to determine which material has a higher retention capacity for streptococci. No differences were observed in the adherence of *S. mutans* to stainless steel, ceramic, or plastic brackets (Papaioannou et al., 2007). Adhesion of cariogenic streptococci was significantly higher for bonding adhesives than for bracket materials, and adhesion to resin-modified glass ionomer was the highest (Lim et al., 2008). Moreover, the levels of *S. mutans* in whole saliva of orthodontically treated patients do not seem to be significantly different between conventional and self-ligating brackets (Pandis et al., 2010). It seems that the material comprising the brackets does not significantly impact on the number of bacteria (Papaioannou et al., 2012). The presence of a salivary pellicle and other bacterial species would seem to have a

significant effect on the adhesion of *S. mutans*, reducing their numbers and further limiting any differences between types of brackets (Papaioannou et al., 2007).

The popularity of orthodontic treatment with thermoplastic aligners has grown recently due to increased demand for esthetic orthodontic appliances. Traditionally, these materials have been used extensively in the form of vacuum-formed retainers after the completion of orthodontic treatment. It has been reported that these retainers influence the adhesion of *S. mutans* and *Lactobacillus* spp., whose numbers of colonies increase two months after debonding (Türköz et al., 2012). However, evidence on the use of thermoplastic aligners as an alternative to fixed appliances is scarce. There is some evidence that recessed and sheltered areas of the aligner, such as the cusp tips and attachment dimples, harbor more biofilm than their flat surfaces (Low et al., 2012). A recent systematic review of the literature published up to 2014 indicated that orthodontic treatment with thermoplastic aligners might be superior in terms of periodontal health, as well as quantity and quality of plaque compared to conventional fixed appliances (Rossini et al., 2015). Additionally, a retrospective study indicated that the periodontal parameters of patients treated with thermoplastic aligners might be better than those treated with lingual fixed appliances (Miethke, & Brauner, 2006). On the other side, a recent randomized trial (Chhibber et al., 2018) found that although patients treated with thermoplastic aligners had better periodontal parameters than patients treated with conventional or self-ligating fixed appliances, ultimately appliance choice had no significant effect overall on periodontal health during treatment. However, to our knowledge no studies have assessed the effect of orthodontic appliances on microbial colonization, which might have a direct influence on both caries and demineralization.

Therefore, this prospective comparative cohort study aimed to answer the following research question: Is there a difference in the salivary prevalence of cariogenic bacteria (*S. mutans, S. sanguinis, L. acidophilus*) among 12-18 year old adolescents treated orthodontically with thermoplastic aligners or fixed appliances?

Methods

Study sample

The sample for this study was recruited from patients presenting for treatment in the postgraduate clinic of the Departments of Orthodontics, School of Dentistry University of Athens and the orthodontic Department

of the 251 Air Force General Hospital, Athens, Greece between September 2014 and July 2016. The following eligibility criteria were used to select appropriate patients to include in this study: adolescent patients aged 12-18 years old of any sex with no reported oral habits detrimental to periodontal health, including smoking, systemic diseases, or any medication 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 2 groups: (i) treatment with conventional fixed appliances and Nickel-Titanium (NiTi) archwires in both arches (metallic labial brackets/tubes, Microarch and Sentalloy Wire 0.014-inch—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 one month. Aligner were used for one month experimentally and the patients were afterwards treated with conventional 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 (Pandis et al., 2010) that reported mean log-*S. mutans* counts per millilitre 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) and standard elastomeric ligature ties were used on incisors, canines, and premolars (molded “O” rings, Ormco, CA, USA). 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 hours 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-PII), 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 (World Health Organization, 1997). 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 minutes 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 were 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*), 300μl of stimulated saliva were 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 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 normalize skewed distributions, the s-PII and s-GI were transformed with their square root, while microbiological counts were transformed with their fifth root. Descriptive statistics were calculated including absolute/relative frequencies for binary variables, means with SDs for normally distributed continuous variables, and medians with interquartile ranges (IQR) for non-normally distributed continuous variables. Differences between groups for normally and non-normally distributed continuous outcomes were assessed with t-tests for independent samples and Mann-Whitney tests, respectively. Differences in the identification frequency of the bacteria at each time point were assessed with Fisher's exact test.

Initial crude linear regression models were built with the transformed outcome as dependent variable, while experimental group (aligner or bracket) and time (T0, T1, and T2) were entered as independent variables. Subsequently, patient age, sex, and oral hygiene (through the s-PII at T0) were added in the initial model one at a time, and if $P \leq 0.20$, they were ultimately added to an adjusted model to account for confounders and including an interaction term of time with appliance. All analyses were run in Stata SE 14.2 (StataCorp LP, College Station, TX) with a two-sided alpha of 5% and an openly provided dataset [21].

Five patients were randomly chosen and their s-GIs re-measured by the same investigators (IS and AP) after 1 month for intra- and inter-examiner repeatability. Repeatability and agreement of the

measurements were assessed with the concordance correlation coefficient [22] and the Bland and Altman [23] method.2 (StataCorp LP, College Station, TX) with a two-sided alpha of 5%.

Results

Clinical parameters

At T0 the thermoplastic aligner and bracket group were comparable for most characteristics, including gender, age, salivary flow rate and DMFT (Table 1). The only exceptions were the periodontal parameters, assessed through the s-PII and the s-GI, where both were higher in the bracket group compared to the aligner group, although only the latter was statistically significant (Table 2).

Although the s-PII was initially similar in the two groups at T0, statistically significant differences were seen between the aligner and the bracket group at T1 and T2 (Table 2). Regression analysis indicated that many factors were significantly associated with s-PII (Table 3), including patient gender (where male patients had higher s-PII than female patients) and initial s-PII at T0 (where patients with initially high s-PII, continued to do so). Apart from these, patients with aligners had statistically significantly lower s-PII throughout treatment than patients with brackets ($P<0.001$). Additionally, the interaction term of time with appliance was close to significance ($P=0.08$), which was further explored by stratified analyses (Appendix 1) and indicated that s-PII variation through time differed between aligner patients (where it tended to decrease through time) and bracket patients (where it tended to increase through time).

For the s-GI on the other side, a significant difference between the two groups was seen at T0, which tended to diminish through time (Table 2). Regression modelling indicated that aligner patients had lower s-GI scores compared to bracket patients and that patients with worse oral hygiene (judged by baseline s-PII) had higher GI scores throughout treatment. On the other hand, no clear variation of s-GI through time was seen, nor any interaction of time with appliance.

Microbiological parameters

As far as qualitative changes are concerned, no differences in the identification of *S. sanguinis, L. acidophilus* and *S. mutans* in the saliva of patients treated with aligners or brackets were found (Table 4). As far as quantitative microbiological parameters are concerned, these could be assessed only for S.

sanguinis and S. mutans, as almost no *L. acidophilus* were identified in the collected saliva samples (Table 5).

The counts of *S. sanguinis* were significantly higher among bracket patients compared to aligner patients both at baseline and through orthodontic treatment (Table 5). Regression analysis indicated that aligner patients had significantly lower counts of *S. sanguinis* than bracket patients (Table 6). Additionally, there was a variation in the *S. sanguinis* counts during the observation period of T0 to T2, with a tendency to differ between aligner and bracket patients (P for interaction=0.11). This was further explored by stratified analyses (Appendix 1) and indicated a variation pattern of *S. sanguinis* that was similar to that of s-PII: the *S. sanguinis* counts showed a tendency to reduce through time among the aligner patients, while *S. sanguinis* counts tended to increase through time among the bracket patients.

Finally, S. mutans counts were similar between the two groups at all times (Table 5), which was further confirmed by the regression analyses (Table 6). There was a small tendency for S. mutans counts to reduce during T0 to T1 (P=0.04), but this faded at T2 and no different variation pattern was seen between the two groups (P for interaction=0.67).

Discussion

The aim of the present prospective cohort study was to compare the salivary levels of cariogenic bacteria among patients treated with either thermoplastic aligners or fixed appliances. The results indicated that patients in the thermoplastic aligner group tended to have lower salivary *S. sanguinis* levels than patients in the fixed appliance group (Table 6). On the other side, no significant differences were seen concerning *L. acidophilus* and *S. mutans*. Oral microbiota attachment in orthodontic patients has been mainly associated with increased risk of S. mutans and lactobacilli colonization, among other species, thus initiating a series of events, which may lead to the development of demineralizations or caries (Gorelick et al., 1982; Øgaard et al., 1988).

As far as periodontal parameters of the two groups compared are concerned, a statistically significant difference in both plaque scores (s-PII) and gingivitis (s-GI) was found between fixed appliances and thermoplastic aligners, which favored the latter (Table 2-3). This agrees with previous data indicating that teenagers treated with aligners display better compliance with oral hygiene, less plaque, and fewer gingival inflammatory reactions than patients with fixed appliances (Abbate et al., 2015). The

ease of oral hygiene maintenance with the clear aligners most likely allows patients to maintain, or possibly even improve, their oral hygiene. A recent systematic review pointed out that a periodontal health indexes are significantly improved during clear aligner treatment, in particular when these appliances were compared to fixed appliances. However, the level of evidence was moderate for all the included studies (Rossini et al., 2015). Additionally, oral hygiene was significantly associated with patient sex, with male patients having significantly higher plaque scores than female patients (Table 3). Furthermore, pre-treatment oral hygiene levels were significantly associated with plaque scores and gingivitis during treatment (Table 3). Finally, no clear variation pattern of oral hygiene was seen through time, which agrees with Clements et al. (2003), who demonstrated that the mean average papillary bleeding scores did not change in a statistically significant manner during aligner treatment.

In the present study instructions were given to brush the thermoplastic appliances once daily. However a recent study demonstrated that the use of a vibrating bath with cleaning solution protocol reduced biofilm adherence more than regular brushing or immersion of the aligner in chlorhexidine mouthwash (Shpack et al., 2014). The use of a chlorhexidine mouthwash as an adjunct to oral hygiene at home does not seem to be necessary for patients undergoing Invisalign treatment, at least for the first 8 months of treatment (Schaefer & Braumann, 2010).

Additionally, several appliance-related factors might influence the intraoral performance of thermoplastic aligners. The material used for the fabrication of the thermoplastic aligners in this study was PET-G, which is the most widely used material for the fabrication of both aligners and retainers (Alexandropoulos et al., 2015). The material used for the Invisalign (Align Technology, Santa Clara, Calif) aligners is polyurethane-based and seems to have higher hardness and modulus values, a slightly higher brittleness, and lesser creep resistance compared with the PETG-based products (Alexandropoulos et al., 2015). However, no evidence on their microbiological colonization exists to our knowledge. It has been suggested that the surface morphology of the aligner might contribute to bacterial adhesion and thereby salivary bacteria levels. The surface of aligners is not completely smooth, but exhibits microabrasions and irregularities and this configuration with its furrowed corrugated facade makes the appliance more conducive to bacterial and biofilm accumulation (Low et al., 2011).

Furthermore, the gingival coverage of an aligner, which differs across the various systems, might directly influence periodontal parameters and microbial colonization. Invisalign aligners have no significant

gingival coverage, however other aligner systems are trimmed to overlap the attached gingiva, in order to improve retention. This method is claimed to provide improved aligner retention but may have periodontal implications. The aligners used in the present study were vacuum formed and cut 2 mm higher than the gingival margin. The manufacturing process may also play in important role, pressure-forming involves higher pressures than vacuum-forming, which might affect up to a limit the detail of the inner, fitting surface of the aligner (Weir, 2017).

Finally, the use of bonded attachments during treatment with thermoplastic aligners might provide additional plaque retentive surfaces on the patient's teeth and thereby increase the microbial load intraorally. However, no such bonded attachments were used in any patients of the present study and therefore the results of this study might not fully reflect cases were multiple irregular attachments are bonded on the teeth to improve the aligner efficacy (Simon et al., 2014).

Even though the present study provides up to now missing evidence on the microbiological performance of orthodontic thermoplastic aligners, it also has some limitations. It is important to note that patients in the present study were followed for a short term of one month. Another study evaluating the effects of fixed appliances indicated that both periodontal health and subgingival plaque composition deteriorated from appliance insertion to the first three months, but then improved during the subsequent three months (Ristic et al., 2007). A similar finding was seen by Karkhanechi et al. (2013) who found an initial deterioration of periodontal parameters after fixed appliance insertion that improved after 6 months of treatment. Additionally, a recent randomized trial (Chhibber et al., 2018) found that although aligner patients tended to have better plaque and gingival bleeding scores that fixed appliance patients in the short-term, no difference could be found for the whole treatment duration. Therefore, it might well be that the short-term salivary levels of cariogenic bacteria observed in this study might not reflect the long-term results. Moreover, only adolescent patients were included in this study and therefore its results might not be generalizable to adult patients. Finally, the present prospective study was not randomized and might be prone to some bias (Papageorgiou et al., 2015).

Conclusion

Within the limitations of the present short-term prospective study, lower salivary levels of S. sanguinis were found in adolescent patients treated for one month with thermoplastic aligners compared to those

treated with conventional fixed appliances. On the other hand, no differences could be found regarding *L. acidophilus* and *S. mutans*.

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Tables

Table 1 Descriptive data of the included sample

Variable	Aligners	Brackets
Female - n (%)	8 (53%)	9 (60%)
Male - n (%)	7 (47%)	6 (40%)
Age - mean (SD)	13.9 (2.0)	13.6 (1.5)
mls - median (IQR)	6.0 (4.0-10.0)	7.0 (3.9-10.0)
DMFT - median (IQR)	0 (0-0)	0 (0-2.0)
s-PII at T0 - median (IQR)	24.0 (21.0-38.0)	30.0 (21.0-44.0)
s-GI T0 - median (IQR)	21.0 (12.0-25.0)	31.0 (19.0-47.0)

DMFT decayed missing filled teeth, *IQR* interquartile range, *SD* standard deviation, *s-GI* simplified gingival index, *s-PII* simplified plaque index

Table 2 Plaque and gingival indices and testing with t-test

Outcome	**Aligner**	**Bracket**	**P***
s-PII T0 (transformed)	**mean (SD)**	**mean (SD)**	
T0	5.34 (0.87)	5.55 (1.08)	0.56
T1	3.97 (1.29)	5.72 (1.24)	0.001
T2	4.80 (1.48)	6.15 (1.79)	0.03
s-GI T0 (transformed)			
T0	4.35 (0.82)	5.77 (1.56)	0.004
T1	4.23 (1.29)	5.71 (1.80)	0.01
T2	5.03 (1.66)	5.81 (1.69)	0.21

* p value for differences between experimental groups (aligner versus bracket) from t-test.
SD standard deviation, *s-GI* simplified gingival index, *s-PII* simplified plaque index

Table 3 Linear regressions with simplified plaque index or gingival index (both square root transformed) as dependent variable

			Crude model			Adjusted model	
Outcome	**Factor**	**Group**	**b (95% CI)**	**P**		**b (95% CI)**	**P**
s-PII*							
	Appliance	Brackets	Referent			Referent	
		Aligners	-1.11 (-1.73 to -0.48)	0.001		-1.05 (-1.55 to -0.55)	<0.001
	Age (per year)		NT			-0.09 (-0.23 to 0.06)	0.25
	Gender	Female	NT			Referent	
		Male				0.56 (0.01 to 1.11)	0.05
	Time	T0	Referent			Referent	
		T1	-0.59 (-1.14 to -0.05)	0.03		-0.59 (-1.14 to -0.05)	0.03
		T2	0.04 (-0.65 to 0.72)	0.92		0.04 (-0.65 to 0.72)	0.92
	s-PII at T0		NT			0.35 (0.12 to 0.58)	0.003
s-GI†							
	Appliance	Brackets	Referent			Referent	
		Aligners	-1.23 (-2.01 to -0.45)	0.002		-1.16 (-1.89 to -0.43)	0.002
	Age (per year)		NT			NT	
	Gender	Female	Referent			Referent	
		Male	NT			NT	
	Time	T0	Referent			Referent	
		T1	-0.09 (-0.59 to 0.41)	0.72		-0.09 (-0.59 to 0.41)	0.72
		T2	0.36 (-0.36 to 1.07)	0.33		0.36 (-0.36 to 1.07)	0.33
	s-PII at T0		NT			0.34 (0.04 to 0.64)	0.02

* The following confounders had P≤0.20 in initial separate models and were added in the adjusted model: age, gender, and s-PII at T0. No significant interaction was found for time with appliance type (P=0.08).

† The following confounder had P≤0.20 in initial separate models and was added in the adjusted model: s-PII at T0 (age and gender had P>0.20 and were not added). No significant interaction was found for time with appliance type (P=0.37).

b unstandardized regression coefficient, *CI* confidence interval, *NT* not tested, *s-GI* simplified gingival index, *s-PII* simplified plaque index

Table 4 Cross-tabulation of positive organisms' findings (binary yes/no variable) and Fisher-exact tests

Bacteria	**Aligner**	**Bracket**	**P**
L. acidophilus	**n/N (%)**	**n/N (%)**	
Present T0	0/15 (0%)	0/15 (0%)	NC
Present T1	0/15 (0%)	1/15 (7%)	1.00
Present T2	0/15 (0%)	1/15 (7%)	1.00
S. sanguinis	**n/N (%)**	**n/N (%)**	
Present T0	15/15 (100%)	15/15 (100%)	NC
Present T1	15/15 (100%)	15/15 (100%)	NC
Present T2	15/15 (100%)	15/15 (100%)	NC
S. mutans	**n/N (%)**	**n/N (%)**	
Present T0	13/15 (87%)	14/15 (93%)	1.00
Present T1	12/15 (80%)	12/15 (80%)	1.00
Present T2	12/15 (80%)	14/15 (93%)	0.60

n patients with event of interest, *N* patients assessed, *NC* non-calculable

Table 5 Bacterial counts for each species (5th root-transformed) at each time point and group with between-group testing with Mann-Whitney test (*) or t-test for independent samples (+), according to normality of data

Bacteria		**Aligner**		**Bracket**	**Test**	**P**
L. acidophilus (transformed)	**n**	**median (IQR)**	**n**	**median (IQR)**		
Count at T0	15	0 (0-0)	15	0 (0-0)	*	1.00
Count at T1	15	0 (0-0)	15	0 (0-0)	*	0.76
Count at T2	15	0 (0-0)	15	0 (0-0)	*	0.76
***S. sanguinis* (transformed)**	**n**	**mean (SD)**	**n**	**mean (SD)**		
Count at T0	15	23.93 (11.67)	15	32.05 (5.24)	+	0.02
Count at T1	15	21.32 (10.55)	15	41.08 (10.02)	+	<0.001
Count at T2	15	22.43 (9.49)	15	34.75 (7.63)	+	0.001
S. mutans (transformed)	**n**	**mean (SD)**	**n**	**mean (SD)**		
Count at T0	15	9.44 (6.06)	15	12.20 (7.24)	+	0.27
Count at T1	15	8.60 (6.05)	15	11.02 (7.73)	+	0.35
Count at T2	15	8.87 (6.14)	15	11.09 (6.71)	+	0.35

IQR interquartile range, *NT* not tested, *SD* standard deviation

Table 6 Linear regressions with S. sanguinis or S. mutans counts (transformed) as dependent variable. The initial crude model coincided with the adjusted model, as no covariates were finally added

Bacteria	**Factor**	**Group**	**b (95% CI)**	**P**
S. sanguinis*	Appliance	Brackets	Referent	
		Aligners	-13.40 (-19.19 to -7.62)	<0.001
	Age	NT		
	Gender	NT		
	Time	T0	Referent	
		T1	3.21 (-0.33 to 6.76)	0.08
		T2	0.60 (-2.07 to 3.26)	0.66
	s-PII at T0	NT		
S. mutans†	Appliance	Brackets	Referent	
		Aligners	-2.47 (-6.99 to 2.05)	0.28
	Age	NT		
	Gender	NT		
	Time	T0	Referent	
		T1	-1.01 (-1.96 to -0.07)	0.04
		T2	-0.84 (-2.08 to 0.40)	0.18
	s-PII at T0	NT		

* The following confounders had P>0.20 in initial separate models and were not added in the adjusted model: age, gender, and s-PII at T0. No significant interaction was found for time with appliance type (P=0.11).
† The following confounders had P>0.20 in initial separate models and were not added in the adjusted model: age, gender, and s-PII at T0. No significant interaction was found for time with appliance type (P=0.67).
CI confidence interval, *NT* not tested, *s-PII* simplified plaque index, *b* unstandardized regression coefficient

Table 7.
Appendix 1 Explorative regression analyses with simplified plaque index or S. sanguinis counts as dependent variable and stratified by appliance subgroup

			Aligners		Brackets		
Outcome	**Factor**	**Group**	**b (95% CI)**	**P_{SG}**	**b (95% CI)**	**P_{SG}**	**$P_{interaction}$**
s-PII	Age	per year	-0.06 (-0.23 to 0.11)	0.47	-0.18 (-0.43 to 0.07)	0.16	0.08
	Gender	Female	Referent		Referent		
		Male	0.25 (-0.61 to 1.10)	0.57	0.95 (0.23 to 1.67)	0.01	
	Time	T0	Referent		Referent		
		T1	-1.37 (-2.04 to -0.69)	<0.001	0.18 (-0.48 to 0.84)	0.60	
		T2	-0.54 (-1.29 to 0.21)	0.16	0.61 (-0.49 to 1.71)	0.28	
	s-PII at T0	per unit	0.53 (0.16 to 0.89)	0.005	0.20 (-0.13 to 0.54)	0.24	
S. sanguinis	Age	per year	NT		NT		0.11
	Gender	Female	NT		NT		
		Male	NT		NT		
	Time	T0	Referent		Referent		
		T1	-2.61 (-4.89 to -0.33)	0.03	9.03 (3.71 to 14.35)	0.001	
		T2	-1.50 (-4.47 to 1.46)	0.32	2.70 (-1.58 to 6.97)	0.22	
	s-PII at T0	per unit	NT		NT		

b unstandardized regression coefficient, *CI* confidence interval, *NT* not tested, $P_{interaction}$ p value for time-differences between appliance subgroups (interaction time with appliance), P_{SG} p for effects within each subgroup, *s-PII* simplified plaque index