



Original article

Real time polymerase chain reaction analysis in the patients treated with fixed appliances after the orthodontic treatment: A follow-up study

Daniah Hassan Alkhayyat^{a,b,*}, Deema Ali Alshammery^b^a Orthodontic resident, Directorate of Health Affairs in Taif Governorate, Taif, Saudi Arabia^b Riyadh Elm University, Riyadh, Saudi Arabia

ARTICLE INFO

Article history:

Received 31 May 2021

Revised 26 June 2021

Accepted 27 June 2021

Available online 6 July 2021

Keywords:

qPCR

Salivary cariogenic bacteria

Orthodontic treatment

Fixed appliances

ABSTRACT

This study aimed to compare the changes in the salivary cariogenic bacteria levels using qPCR and oral hygiene status after orthodontic treatment with fixed appliances during the retention phase concerning the patient and treatment variables. In this study, saliva samples were collected from 35 patients before debonding (T_0) and after five weeks of debonding on retention (T_1). The saliva samples were collected to extract the genomic DNA, and using specific probes and primers using real-time polymerase chain reaction was performed to analyze the changes in *S. mutants*, *S. sobrinus*, *L. Casei* after orthodontic treatment with fixed appliances. Additionally, OHI levels were also measured. The current study confirms the statistical association between T_0 and T_1 groups of *S. mutants* ($p = 0.028$) and *S. sobrinus* ($p = 0.049$). However, a lack of association was observed with *L. Casei* ($p > 0.05$). The number of bacteria was decreased from the T_0 group and increased in the T_1 group in Streptococcus mutants (*S. mutants*) and Streptococcus Sobrinus (*S. sobrinus*) while in Lactobacillus Casei (*L. Casei*) it was vice versa between T_0 and T_1 groups. The Oral Hygiene Levels (OHI) levels were also found to be statistically associated ($p = 0.003$). This study concludes that comparing the salivary cariogenic bacterial levels at T_0 (before debonding of fixed orthodontic appliances), with T_1 (Five weeks after the debonding), and despite better oral hygiene, there was increase in salivary *S mutants and S sobrinus levels*. The current study suggested that orthodontic patients need careful hygienic procedures during the retention period. Future studies are recommended with additional follow-up and a large sample size.

© 2021 The Author(s). Published by Elsevier B.V. on behalf of King Saud University. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

1. Introduction

Orthodontic brackets adhere to the tooth surface, which makes the ability of the patient to clean his teeth more difficult and thereby increases the biofilm formation and gives new possibilities for microorganisms to attach themselves (Baka et al., 2013; Fatani et al., 2017).

Studies have shown that cariogenic microorganisms, including *mutans streptococci*, which play a significant role in initiating carious lesions, and *lactobacilli*, which may be more noted in their progression, increase the dental plaque and saliva of patients after the

bonding of orthodontic appliances (Baka et al., 2013; Peros et al., 2011; Van Houte, 1994; Abushaheen et al., 2020).

Therefore, knowing the bacterial changes in patients who had orthodontic treatment, including the retention phase, is significant. Observing changes in the microbial composition might help guide the prevention and treatment of caries (Lucchese and Gherlone, 2013). The retention phase of orthodontic treatment is where dental movements are stabilized after active treatment. It is crucial for the longevity of orthodontic treatment results. Multiple fixed and removable retention methods are routinely used in clinical practice, but they have advantages and disadvantages. Fixed retainers make oral hygiene procedures more difficult and enhance the accumulation of plaque and calculus, while removable retainers cover tooth surfaces and interrupt the flushing effect of saliva (Eroglu et al., 2019).

Different methods have been used to identify *Streptococcus mutans* and *lactobacilli* in saliva. Recently, real-time polymerase chain reaction (rt-PCR/qPCR) has appeared as a more rapid and sensitive method of quantifying and detecting specific bacterial species. The qPCR can detect absolute numbers of targeted

* Corresponding author.

E-mail address: daniah.h.alkhayyat@student.riyadh.edu.sa (D.H. Alkhayyat).

Peer review under responsibility of King Saud University.



bacteria, with various applications including amplification, measurement, and quantification that can be managed simultaneously to minimize the chances of contamination (Jung et al., 2014). Most studies have evaluated the changes in *S. mutans* and *Lactobacillus* levels during orthodontic treatment with fixed or removable appliances; however, microbiologic data on the retention phase after active orthodontic treatment are limited (Eroglu et al., 2019; Jung et al., 2014; Rosenbloom and Tinanoff, 1991). While the changes in cariogenic bacteria during orthodontic treatment have been considerably evaluated, few studies have evaluated changes in these bacteria after orthodontic treatment at the retention stage. Considering the importance of *Streptococcus mutans* (*S. mutans*) and *Streptococcus Sobrinus* (*S. sobrinus*) while in *Lactobacillus Casei* (*L. Casei*) levels in detecting the risk of enamel demineralization and caries, this current research was undertaken (Eroglu et al., 2019; Jung et al., 2014; Rosenbloom and Tinanoff, 1991).

Limited studies were carried out in the global population and there are no studies have been implemented in the Saudi population. So, the current study was carried out in the capital city of the kingdom. The aim of this study was to compare the changes in the salivary cariogenic bacteria levels using qPCR and oral hygiene status after orthodontic treatment with fixed appliances during the retention phase concerning the patient and treatment variables.

2. Materials and methods

2.1. Sample enrollment

The first procedure in this study was to obtain ethical approval (FPGRP/2020/472/233/229) in order to carry out the research at Riyadh Elm University (REU). In this study, we obtained information from 35 patients who completed orthodontic treatment with fixed appliances at Department of Orthodontics at REU Hospital and a couple of private clinics. A 0.022-in slot MBT bracket system was used to treat all patients (3 M unitek, USA). This prospective clinical study comprised both genders (21 female and 14 male) with ages ranging from 15 to 35 years old, with participants or their guardians consenting their participation by signing the informed consent form. The inclusion criteria for selecting the patient include permanent dentition with a minimum treatment term of 12 months and treatment with fixed orthodontic appliances. Exclusion criteria for this study subjects include smoking, short-term or long-term antibiotic usage, topical fluoride application, and Patients with periodontitis and patients who reported having received any sort of periodontal therapy during the last 6 months, patients with any of the systemic diseases, or patients with active carious lesions.

2.2. Saliva collection

In this study, 70 saliva samples were collected from the 35 orthodontic patients. From each patient, the saliva sample was collected twice, on day 1 (T_0) before debonding and five weeks after debonding, and on retention (T_1). From each patient, an unstimulated whole saliva sample was collected in sterile tube using the Oragene tube (DNA genotek, Ottawa, Canada) following the manufacturer's instructions (Alharbi et al., 2020). Oragene commonly used a sophisticated technique wherein preservation buffers are used to protect the integrity of the sample and stabilizes samples at the point of collection at room temperature until processing and extraction take place (Sindhu and Saliva, 2014). Saliva collections were made during morning hours, between 8 and 11 am. The subjects were in the fasting state or two hours after breakfast. Patients were recommended not to eat and drink, or brush their

teeth or gargle before 30 mins to 60 mins of saliva sample collection to avoid contamination. The 2 mL of the saliva sample was collected in the spitting method (Priya and Prathibha, 2017). The participants were asked to rinse the mouth out well for 1 min with distilled drinking water then to expectorate the water. Five minutes after this oral rinse, the subjects were asked to spit into the Oragene tube. They were recommended to sit on the dental chair with the position of the right side. The participants were instructed to stop talking and to lower their heads, allowing saliva to flow naturally to the front of their mouths. They were also asked not to cough up mucus as saliva is collected.

2.3. Extraction of DNA using saliva

A total of 2 mL of saliva was collected from each patient and saliva was used to extract the genomic DNA using the Oragene DNA isolation kit (Alshammery and Khan, 2021). Nanodrop was used to measure the quality of DNA. Simultaneously, all of the DNA samples were loaded into a 0.8% agarose gel for the re-confirmation (Khan et al., 2019). To continue with the qPCR analysis, all of the genomic DNA was transformed into 10 ng of DNA. In this study, we used three bacterial markers (*L. Casei*, *S. Mutants*, & *S. Sorbinus*) to detect and quantify carcinogenic microorganisms. TaqMan genotyping was performed for *L. Casei* and SYBR green PCR was performed for *S. Mutants* and *S. Sorbinus*. The details of the annealing temperature, band sizes, primers and probes were shown in Table 1. In this study, FAM and TAMRA probes were used and qPCR analysis was performed (Alharbi et al., 2019; Alharbi et al., 2021). In this study, we used 25.0 μ l of master mix, 10 ng of 5.0 μ l of genomic DNA, 1.0 μ l of probes, reversed and forward oligonucleotide sequences. The final concentration was diluted using 17.0 μ l of double distilled water. The experiment was carried out in a 7500 ABI rt-PCR machine with a total of 50- μ l reaction. The obtained data was transported into excel and used for the statistical analysis.

2.4. Statistical analysis

The statistical analysis was performed using Microsoft Excel Software. The Shapiro-Wilk test was used to assess data normality and *t*-test was obtained to find differences in bacterial numbers and OHI-S at T_0 and T_1 using SPSS software (Version 25.0). The bacterial count was measured using mean \pm standard deviation (SD) in both the male and female participants. One-way analysis of variance (ANOVA) was performed with a minimum of three or more than three per group. Values were considered to be statistically significant at $p < 0.05$ (Khan et al., 2015).

3. Results

3.1. Patients details

In this study, 35 patients were involved who finished their orthodontic treatment and ready to start the retention phase. The

Table 1
Describes the details of the annealing temperature, band sizes, primers and probes.

Bacteria	Oligonucleotide Sequence	Annealing	Base Pairs
<i>L. Casei</i>	F: CTATAAGTAAGCTTTGATCCGGAGATTT	60 °C	132 bp
	R: CTTCTGCGCGTACTGAGATGT		
	P: ACAAGCTATGAATTCACATATGC		
<i>gtfB</i> (<i>S. Mutants</i>)	F: CTACACTTTCCGGTGGCTTG	60 °C	261 bp
	R: GAAGCTTTTACCATTAGAAGCTG		
<i>gtfU</i> (<i>S. Sorbinus</i>)	F: AAAACATTGGGTTACGATTCCG	60 °C	156 bp
	R: CGTCATTGGTAGTAGCCTGA		

retention phase was categorized into T₀, which represents the day of debonding of treatment (directly before debonding), and T₁ defines as five weeks after debonding of treatment (on retention). Prior to debonding, orthodontic treatment with fixed appliances lasted anywhere from one year to seven years and six months. Among 35 patients, 60% of patients were female and 40% were males. The mean age of the 35 participants was in the range of 21.5 ± 5.8. The mean age of female participants was 20.5 ± 6.2 and male participants were 23 ± 4.9. The minimum age of male participants was 15 years and the maximum age was 34 years who participated in this study. Among women, the minimum and maximum ages were 14 years and 39 years. In this study, two types of retainers were enrolled and among the participants, Hawley with lingual fixed retainer (57.2 %), Hawley without lingual fixed retainer (42.8 %). The details were documented in Table 2. The mean of the treatment duration was found to be 2.7 ± 1.6 and among males, the estimated time was 2.9 ± 1.9 and in females was 2.5 ± 1.4 (Fig. 1).

3.2. Bacterial count

Table 3 describes the qPCR analysis performed with Taqman genotyping for *L. Casei* and SYBR green PCR with *S. Mutants* and *S. Sobrinus*. The mean bacterial count present in the *L. Casei* within the T₀ group is 27.08 ± 4.20 and in the T₁ group, the mean value was documented as 26.16 ± 4.81. When a paired sample *t*-test was performed between T₀ and T₁, the *p*-value was found to be inconsistent (*p* = 0.100). The minimum bacterial count documented in the T₀ group *L. Casei* was 17.54 and 39.73 was the maximum count. Simultaneously, the minimum bacterial count in T₁ group was 18.12 and the maximum bacterial count was 43.67 with *L. Casei*. The T₁ group was documented with the highest bacterial count when compared with T₀ group. The *t*-statistics were found to be 1.689 and 0.92 was documented with the mean difference. The upper and lower limits were 2.03 and -0.18 levels.

SYBR green PCR was performed for *S mutants* and *S sobrinus* in the patients before and after the orthodontic treatment. The 50 µl reaction of SYBR green PCR confirms the *S mutants* was shown to be statistically associated when compared the bacterial count between T₀ and T₁ groups (*p* < 0.05). In the T₀ group, the mean value of *S mutants* was documented to be 28.60 ± 3.05 and in T₁ group, 30.56 ± 3.41 group mean values were found. The paired sample *t*-tests were found to be a statistical association when compared between T₀ and T₁ groups (*p* = 0.028). The *t*-statistics were document to be -2.303 along with -1.96 as mean differences. Both -0.23 and -3.69 were confirmed as upper and lower limits obtained in this study when compared between T₀ and T₁ groups. The 35.25 ± 4.47 was the obtained bacterial count mean value in T₀ group for *S sobrinus* and 36.97 ± 2.71 was the documented bacterial

Table 2
Details of the involvement of orthodontic patients.

	Orthodontic treatment (n = 35)	Prevalence (%)
Age	21.5 ± 5.80	NA
Gender: Male	14	40%
Female	21	60%
Type of retainer	35	100%
Hawley with lingual fixed retainer	20	57.2 %
Hawley without lingual fixed retainer	15	42.8 %
Treatment Duration	2.71 ± 1.61	NA
Treatment duration in males	2.95 ± 1.90	NA
Treatment duration in females	2.55 ± 1.41	NA

NA = Not applicable.

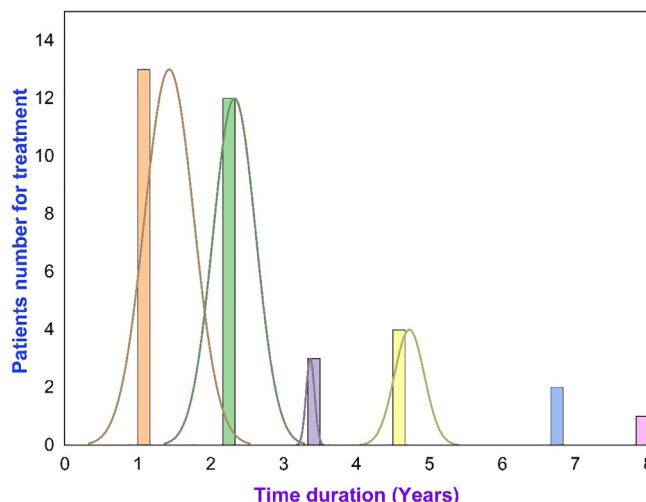


Fig. 1. The orthodontic treatment within the patients involved in this study.

count in the T₁ group. Additionally, the upper and lower limits were documented as -0.01 and -3.43. The mean difference and *t*-statistics were confirmed to be -1.72 and -2.044. The statistical analysis showed that there is statistical association between T₀ and T₁ groups (*p* = 0.049).

3.3. Oral hygiene index levels

In this study, OHI levels were measured in patients at T₀ and T₁. The mean value of OHI in T₀ patients was 1.3 ± 0.527 and the minimum value documented in subjects at T₀ was 0.16 and the maximum value was 2.32. In female subjects, the minimum 0.33 and maximum obtained value is 2.32. The mean value for the female subjects was 1.3 ± 0.6 and in male subjects, 0.16 was the obtained minimum value and 1.66 was the maximum value. The mean value for the male subjects was 1.27 ± 0.4. The mean value of OHI in T₁ patients was 0.94 ± 0.52 and the minimum value documented in T₁ patients was 0.25 and the maximum value was 2.5. In female subjects, the minimum obtained value is 0.25 and the maximum obtained value is 2.5. The mean value for the female subjects was 1.0 ± 0.61 and in male subjects, 0.25 was the obtained minimum value and 1.2 was the maximum value. The mean value for the male subjects was 0.8 ± 0.34. When the *t*-tests were performed between the T₀ and T₁ values, a significant statistical association was obtained (*p* = 0.04). The mean difference was documented to be 0.37 and *t*-statistics was found to be 3.172. The upper and lower limit values were 0.61 and 0.13.

3.4. Gender-based comparison of bacterial count and OHI-S

In this study, 60% of confirmed participants were females and 40% of them were males. Table 4 documents gender-based variations in the salivary levels of *L. Casei*, *S. mutants*, *S. sobrinus* and OHI levels. In the T₀ group of *L. Casei*, the mean male values and females' values were found to be 26.12 ± 5.07 and 27.72 ± 3.49 with no statistical association (*p* = 0.27). While in the T₁ group, 25.67 ± 4.67 and 26.49 ± 4.99 was the mean values confirmed from male and female participants (*p* = 0.62). The mean values between male and female participants were compared with *S. mutants* in the T₀ group and found to be 28.95 ± 2.61 and 28.36 ± 3.36 (*p* = 0.58) and in T₁ group was 30.57 ± 4.17 and 30.56 ± 2.92 (*p* = 0.99). While in the *S sobrinus*, the mean values documented between male and female groups in the T₀ group was 36.93 ± 1.70 and 34.12 ± 5.37 (*p* = 0.06) and in the T₁ group, it was 36.64 ± 3.51 and

Table 3
TaqMan and SYBR green PCR analysis for bacterial counting in *L. Casei*, Total bacteria, *S. Mutants* and *S. sobrinus*.

	Groups (n = 35)	Mean	SD	t-statistics	df	Mean difference	Upper limit	Lowe limit	P-value
S. Mutants	T ₀	28.60	3.05	–	–	–	–	–	–
	T ₁	30.56	3.41	–2.303	34	–1.96	–0.23	–3.69	0.028
S. Sobrinus	T ₀	35.25	4.47	–	–	–	–	–	–
	T ₁	36.97	2.71	–2.044	34	–1.72	–0.01	–3.43	0.049
L. Casei	T ₀	27.08	4.20	–	–	–	–	–	–
	T ₁	26.16	4.81	1.689	34	0.92	2.03	–0.18	0.100
OHS-I	T ₀	1.31	0.52	–	–	–	–	–	–
	T ₁	0.94	0.52	3.172	34	0.37	0.61	0.13	0.003

37.19 ± 2.08 (p = 0.56). In the OHI, the T₀ group levels in male and female participants were 1.27 ± 0.39 and 1.34 ± 0.60 (p = 0.70) and in the T₁ group, the mean values between male and female participants were 0.84 ± 0.34 and 1.00 ± 0.61 (p = 0.37). When the comparison was carried out between males and females with both T₀ and T₁ groups showed no differences with *L. Casei*, *S. mutants*, *S. sobrinus*, total bacteria, and OHI levels (p > 0.05).

3.5. Retainer type-based comparison of bacterial count and OHI-S

In this study, Hawley with lingual fixed retainer (57.2 %), Hawley without lingual fixed retainer (42.8 %). Table 4 documents retainer type-based variations in the salivary levels of *L. Casei*, *S. mutants*, *S. sobrinus* and OHI levels. In the T₀ group of *L. Casei*, the mean Hawley with fixed retainer values and Hawley without fixed retainer values were found to be 26.10 ± 3.8 and 28.40 ± 4.47 with no statistical association (p = 0.11). While in the T₁ group, 25.02 ± 3.9 and 27.68 ± 5.51 was the mean values confirmed from participants on Hawley with fixed retainer and Hawley without fixed retainer (p = 0.10). The mean values between Hawley with fixed retainer values and Hawley without fixed retainer participants were compared with *S. mutants* in the T₀ group and found to be 28.35 ± 3.09 and 28.93 ± 3.07 (p = 0.58) and in T₁ group was 30.76 ± 3.63 and 30.30 ± 3.21 (p = 0.69). While in the *S. sobrinus*, the mean values documented between Hawley with fixed retainer and Hawley without fixed retainer groups in the T₀ was 35.47 ± 3.66 and 34.59 ± 5.43 (p = 0.46) and in the T₁ group, it was 36.50 ± 3.09 and 37.60 ± 2.03 (p = 0.24). In the OHI-S, the T₀ group levels in participants on Hawley with fixed retainer and Hawley without fixed retainer were 1.25 ± 0.51 and 1.40 ± 0.54 (p = 0.39) and in the T₁ group, the mean values between Hawley with fixed retainer and Hawley without fixed retainer groups were 0.87 ± 0.44 and 1.02 ± 0.61 (p = 0.42). When the comparison was carried out between Hawley with fixed retainer values and Hawley without fixed retainer with both T₀ and T₁ groups showed no differences with *L. Casei*, *S. mutants*, *S. sobrinus* and OHI-S levels (p > 0.05; Table 5).

3.6. ANOVA analysis

ANOVA was performed in this study using the treatment duration as the primary parameter along with various bacterial counts

Table 4
Gender variation between T₀ and T₁ phases of qPCR bacterial counting and OHI-S.

	Groups (n = 35)	Male Mean	Male S.D	Female Mean	Female S.D	P-value
S mutants	T ₀	28.95	2.61	28.36	3.36	0.58
	T ₁	30.57	4.17	30.56	2.92	0.99
S sobrinus	T ₀	36.93	1.70	34.12	5.37	0.06
	T ₁	36.64	3.51	37.19	2.08	0.56
L. Casei	T ₀	26.12	5.07	27.72	3.49	0.27
	T ₁	25.67	4.67	26.49	4.99	0.62
OHI-S	T ₀	1.27	0.39	1.34	0.60	0.70
	T ₁	0.84	0.34	1.00	0.61	0.37

such as total bacteria, *S mutants*, *S sobrinus*, *L. Casei*, and OHI-S. This analysis was performed in both T₀ (Table 6) and T₁ groups (Table 7). The sample size of the treatment duration was categorized into four groups as (i) 0–1.9 years, (ii) 2.0–2.9 years, (iii) 3.0–4.9 years, and (iv) 5.0–7.9 years. The 37.1% of patients are under 0–1.9 years of treatment group. For the treatment duration between 2.0 and 2.9 years, almost 34.3% of participants were listed. Only 20% of the patients had received the treatment for 3.0–4.9 years and finally, 8.6% of participants had undergone treatment for 5.0–7.9 years. (Tables 6 and 7) describe the relationship between treatment duration and different bacterial count and OHI-S in the T₀ stage and T₁ stages. The mean values for *S. mutants* bacterial count for the four treatment groups in an ascending order according to the treatment duration were 27.86 ± 2.84, 29.93 ± 3.05, 27.01 ± 2.72, and 30.16 ± 3.20. The *S. sobrinus* mean values were 35.60 ± 4.27, 36.71 ± 2.35, 32.78 ± 6.79, and 33.61 ± 4.76. In the *L. Casei*, the mean values were 28.11 ± 2.19, 26.64 ± 4.22, 26.54 ± 7.26, and 25.64 ± 1.60. Finally, the OHI levels were as follows 1.13 ± 0.57, 1.32 ± 0.52, 1.54 ± 0.36, and 1.55 ± 0.58. In this study with the T₀ group, the minimum obtained bacterial count was 25.64 ± 1.60 in *L. Casei* with the treatment duration between 5.0 and 7.9 years and maximum was in *S. sobrinus* with 36.71 ± 2.35 with the treatment duration of 2.0–2.9 years of age. The ANOVA analysis showed a significant association in the T₀ group with *S. sobrinus* (p = 0.03) and *L. Casei* (p = 0.004). However, a negative association was obtained between *S. mutants* (p = 0.98) and OHI (p = 0.69). Table 7 documents with T₁ group and ANOVA analysis showed a negative association with all the bacterial strains and OHI. The *S. mutants* showed 31.82 ± 3.29 during 0–1.9 years of treatment, 29.63 ± 3.84 for 2–2.9 years, 29.51 ± 3.06 for 3.0–4.9 years, and 31.30 ± 1.96 for 5.0–7.9 years of treatment with obtained p-value as 0.73. A similar pattern for *S. sobrinus* was 37.31 ± 2.11, 37.11 ± 3.03, 36.43 ± 3.70, and 36.22 ± 1.83 (p = 0.36) and *L. Casei* was 27.01 ± 3.74, 24.89 ± 3.97, 27.01 ± 8.08, and 25.55 ± 3.13 (p = 0.08). The OHI levels were as follows 1.06 ± 0.67, 0.82 ± 0.43, 0.96 ± 0.36 and 0.80 ± 0.47 (p = 0.31).

4. Discussion

The current study examined the levels of total salivary bacteria, *S. mutans*, *S. sobrinus*, and *L. Casei*, and the level of oral hygiene in

Table 5
Retainer type variations between T₀ and T₁ phases of qPCR bacterial counting and OHI.

	Groups (n = 35)	Hawley w fixed Mean	Hawley w fixed S.D	Hawley w/o fixed Mean	Hawley w/o fixed S.D	P-value
S mutants	T ₀	28.35	3.09	28.93	3.07	0.58
	T ₁	30.76	3.63	30.30	3.21	0.69
S sobrinus	T ₀	35.74	3.66	34.59	5.43	0.46
	T ₁	36.50	3.09	37.60	2.03	0.24
L. Casei	T ₀	26.10	3.8	28.40	4.47	0.11
	T ₁	25.02	3.9	27.68	5.51	0.10
OHI-S	T ₀	1.25	0.51	1.40	0.54	0.39
	T ₁	0.87	0.44	1.02	0.61	0.43

Table 6
Correlation between T₀ phase of various qPCR bacterial counting and OHI-S involved in this study.

Treatment duration	0–1.9 Years	2.0–2.9 Years	3.0–4.9 Years	5.0–7.9 Years	P-value	χ ²
Sample size	13 (37.1%)	12 (34.3%)	07 (20%)	03 (8.6%)	1.00	–
S. mutants (T₀)	27.86 ± 2.84	29.93 ± 3.05	27.01 ± 2.72	30.16 ± 3.20	0.98	0.14
S. sobrinus (T₀)	35.60 ± 4.27	36.71 ± 2.35	32.78 ± 6.79	33.61 ± 4.76	0.03	8.31
L. Casei (T₀)	28.11 ± 2.19	26.64 ± 4.22	26.54 ± 7.26	25.64 ± 1.60	0.004	13.17
OHI-S (T₀)	1.13 ± 0.57	1.32 ± 0.52	1.54 ± 0.36	1.55 ± 0.58	0.69	1.42

Table 7
Correlation between T₁ phase of various qPCR bacterial counting.

Treatment duration	0–1.9 Years	2–2.9 Years	3–4.9 Years	5–7.9 Years	P-value	χ ²
Sample size	13 (37.1%)	12 (34.3%)	07 (20%)	03 (8.6%)	1.00	–
S. mutants (T₁)	31.82 ± 3.29	29.63 ± 3.84	29.51 ± 3.06	31.30 ± 1.96	0.73	1.25
S. sobrinus (T₁)	37.31 ± 2.11	37.11 ± 3.03	36.43 ± 3.70	36.22 ± 1.83	0.36	3.15
L. Casei (T₁)	27.01 ± 3.74	24.89 ± 3.97	27.01 ± 8.08	25.55 ± 3.13	0.08	6.64
OHI (T₁)	1.06 ± 0.67	0.82 ± 0.43	0.96 ± 0.36	0.80 ± 0.47	0.31	3.53

patients who had orthodontic treatment with fixed appliances. The current study results confirmed that *S. mutans* ($p = 0.028$) and *S. sobrinus* ($p = 0.049$) were significantly associated when compared between T₀ and T₁ groups as the numbers of these bacteria increased at T₁ (5 weeks after debonding and on retention). Additionally, OHI-S also had a positive association statistically ($p = 0.003$) as the values decreased at T₁ which indicate improvement of oral hygiene after debonding of fixed appliances. This is because removing the orthodontic equipment removes their plaque-retentive effect, which making maintaining good oral hygiene easier. Furthermore, oral hygiene procedures such as prophylaxis and scaling at debonding can improve oral hygiene status and periodontal conditions immediately. However, our results showed statistically non-significant decrease of *L. Casei* after orthodontic treatment ($p = 0.10$).

In this study, the bacterial count was increased during the T₁ period when compared with T₀ periods in *S. mutans* (30.56 ± 3.41 vs. 28.60 ± 3.05), and *S. sobrinus* (36.97 ± 2.71 vs. 35.25 ± 4.47), and there was a decrease in *L. Casei* (26.16 ± 4.81 vs. 27.08 ± 4.20). we did not find differences in salivary microbiological levels and OHI-S scores between males and females or between patients on removable with fixed retainer and patients on removable retainer only. The retention period was followed up for only five weeks in our study. A previous study (Rosenbloom and Tinanoff, 1991) was one of the first studies which evaluated patients' salivary *S. mutans* levels before, during, and after orthodontic treatment. They found that *S. mutans* levels were significantly elevated during active treatment while they decreased significantly to levels comparable to age-matched untreated controls at 6–15 weeks into the retention phase of treatment. When the patients were no longer wearing any retention appliances, *S. mutans* levels were like the participants in control groups. According to the results of this study, orthodontic care does not result in any long-term increases in salivary levels of *S. Mutans*.

Eroglu et al (Eroglu et al., 2019) compared and evaluated salivary microbial levels using real-time PCR and periodontal clinical parameters in patients using a fixed lingual retainer, a removable vacuum-formed retainer and Hawley retainer after orthodontic treatment with fixed appliances. They concluded that with all retainers, regardless of whether they are fixed or removable, oral hygiene improved after orthodontic treatment with fixed appliances which agree with our findings with no significant differences in salivary bacterial levels or OHI-S between retainer types and the improvement of oral hygiene regardless of retainer type. However, in contrast to our results, the *S. mutans* levels in Eroglu et al (Eroglu et al., 2019) study was decreased significantly after debonding in the retention stage while *L. Casei* salivary levels documented a decrease after debonding similar to our findings.

Our study was in agreement with Jung et al (Jung et al., 2014) which has registered a sharp reduction in oral hygiene index values one week after finishing the orthodontic treatment. In other words, one week after debonding, patients' oral hygiene improved dramatically. Jung et al (Jung et al., 2014) analyzed the changes in salivary *mutans streptococci* after orthodontic treatment using real-time PCR and showed that the total bacteria significantly decreased while salivary *S. mutans* and *S. sobrinus* levels significantly increased after orthodontic treatment, which agrees with our findings regarding *S. mutans* and *S. sobrinus* levels after orthodontic treatment. Fixed orthodontic treatment makes oral hygiene difficult and affects oral flora by growing bacterial retentive areas. This is why, by removing orthodontic instruments, oral hygiene improves (Lara-Carrillo et al., 2010). These previous studies' study periods were limited to the early stages of the retention phase. However, in our research, there were only T₀ and T₁ groups.

The major limitations of this study were that the experimental duration was short, with only two-time points T₀ and T₁ comparisons after orthodontic treatment. One of the limitations was the long treatment duration with fixed orthodontic appliances in 10

subjects, as the length of orthodontic treatment in these patients was more than three years and up to seven years six months long. We included them in the study to increase the sample size and we did not exclude patients with long treatment duration but make it as a parameter to find out if there was any correlation between the treatment duration and the changes in the levels of cariogenic bacteria and the oral hygiene index levels. Another limitation is that we did not standardize the oral hygiene protocol and monitor it for all the subjects during the clinical study.

5. Conclusion

This *in vivo* quantification assay was conducted to see whether levels of salivary *S. mutans*, *S. sobrinus*, *L. Casei*, and OHI-S were stable after orthodontic treatment by performing qPCR in the saliva for microbiological analysis. This study concludes that comparing the salivary cariogenic bacterial levels at T₀ (before debonding of fixed orthodontic appliances), with T₁ (Five weeks after the debonding), and despite better oral hygiene, there was an increase in salivary *S. mutans* and *S. sobrinus* levels. Our results are also consistent, and at the same time, inconsistent with other global studies due to the limited sample size and limited groups. The current study concludes that orthodontic patients need careful hygienic procedures during the retention. Future studies should be follow-up for three months, six months, nine months, and one year of follow-up for longer results and analysis. Thus, additional steps must be taken to maintain oral hygiene and reduce their susceptibility to caries and other periodontal disorders for patients undergoing fixed orthodontic treatment and after orthodontic treatment in the retention stage. Future studies recommend carrying out a large sample size in multiple institutes in all age groups and comparing different retainer types and different retainer designs to rule out the disease. A standardization of oral hygiene protocol for patients during the clinical study needs to be considered in future researches.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

Baka, Z.M., Basciftci, F.A., Arslan, U., 2013. Effects of 2 bracket and ligation types on plaque retention: a quantitative microbiologic analysis with real-time

- polymerase chain reaction. *Am. J. Orthodont. Dentofac. Orthopedics.* 144 (2), 260–267.
- Fatani, E.J., Almutairi, H.H., Alharbi, A.O., Alnakhli, Y.O., Divakar, D.D., Alkheraif, A.A., et al., 2017. In vitro assessment of stainless steel orthodontic brackets coated with titanium oxide mixed Ag for anti-adherent and antibacterial properties against *Streptococcus mutans* and *Porphyromonas gingivalis*. *Microb. Pathog.* 112, 190–194.
- Peros, K., Mestrovic, S., Anic-Milosevic, S., Slaj, M., 2011. Salivary microbial and nonmicrobial parameters in children with fixed orthodontic appliances. *Angle Orthodontist.* 81 (5), 901–906.
- Van Houte, J., 1994. Role of micro-organisms in caries etiology. *J. Dent. Res.* 73 (3), 672–681.
- Abushaheen, M.A., Fatani, A.J., Alosaimi, M., Mansy, W., George, M., Acharya, S., et al., 2020. Antimicrobial resistance, mechanisms and its clinical significance. *Dis. Mon.* 66, (6) 100971.
- Lucchese, A., Gherlone, E., 2013. Prevalence of white-spot lesions before and during orthodontic treatment with fixed appliances. *Eur. J. Orthod.* 35 (5), 664–668.
- Eroglu, A.K., Baka, Z.M., Arslan, U., 2019. Comparative evaluation of salivary microbial levels and periodontal status of patients wearing fixed and removable orthodontic retainers. *Am. J. Orthod. Dentofac. Orthop.* 156 (2), 186–192.
- Jung, W.-S., Kim, H., Park, S.-Y., Cho, E.-J., Ahn, S.-J., 2014. Quantitative analysis of changes in salivary mutans streptococci after orthodontic treatment. *Am. J. Orthod. Dentofac. Orthop.* 145 (5), 603–609.
- Rosenbloom, R.G., Tinanoff, N., 1991. Salivary *Streptococcus mutans* levels in patients before, during, and after orthodontic treatment. *Am. J. Orthod. Dentofac. Orthop.* 100 (1), 35–37.
- Alharbi KK, Al-Sheikh YA, Alsaadi MM, Mani B, Udayaraja GK, Kohailan M, et al. Screening for obesity in the offspring of first-cousin consanguineous couples: A Phase-I study in Saudi Arabia. *Saudi J. Biol. Sci.* 2020;27(1):242–6. Epub 2020/01/01. doi: 10.1016/j.sjbs.2019.09.001. PubMed PMID: 31889843; PubMed Central PMCID: PMC6933162.
- Sindhu, S., Saliva, Jagannathan N., 2014. A cutting edge in diagnostic procedures. *J. Oral Dis.* 2014.
- Priya, K.Y., Prathibha, K.M., 2017. Methods of collection of saliva-a review. *Int. J. Oral Health Dent.* 3 (3), 149–153.
- Alshammery, A.F., Khan, I.A., 2021. Screening of Obese Offspring of First-Cousin Consanguineous Subjects for the Angiotensin-Converting Enzyme Gene with a 287-bp Alu Sequence. *J. Obes. Metab. Syndr.* 30 (1), 63.
- Khan IA, Jahan P, Hasan Q, Rao P. Genetic confirmation of T2DM meta-analysis variants studied in gestational diabetes mellitus in an Indian population. *Diabetes Metab Syndr.* 2019;13(1):688–94. Epub 2019/01/16. doi: 10.1016/j.dsx.2018.11.035. PubMed PMID: 30641791.
- Alharbi KK, Al-Sulaiman AM, Shedaïd KMB, Al-Shangiti AM, Marie M, Al-Sheikh YA, et al. MTNR1B genetic polymorphisms as risk factors for gestational diabetes mellitus: a case-control study in a single tertiary care center. *Ann Saudi Med.* 2019;39(5):309–18. Epub 2019/10/04. doi: 10.5144/0256-4947.2019.309. PubMed PMID: 31580701; PubMed Central PMCID: PMC6832319.
- Alharbi, K.K., Alshammery, A.F., Aljabri, O.S., Khan, I.A., 2021. Relationship Between Serum Amyloid A1 (SAA1) Gene Polymorphisms Studies with Obesity in the Saudi Population. *Diabetes, Metab. Syndr. Obes. Targets Ther.* 14, 895.
- Khan, I.A., Jahan, P., Hasan, Q., Rao, P., 2015. Relationship between PTEN and gestational diabetes in Asian Indians women. *J. Health Spec.* 3 (3), 184.
- Lara-Carrillo E, Montiel Bastida NM, Sánchez Pérez L, Alanís Tavira J. Effect of orthodontic treatment on saliva, plaque and the levels of *Streptococcus mutans* and *Lactobacillus*. 2010.