Impact of Chlorhexidine Pretreatment Followed by Probiotic *Streptococcus salivarius* Strain K12 on Halitosis in Children: A Randomised Controlled Clinical Trial

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Purpose: The aim of this study was to examine the effect of chlorhexidine disinfection, as a chemical method of oral hygiene practice, and subsequent use of probiotics on halitosis in children. The effects of mechanical and chemical oral hygiene practice methods on the severity of halitosis were also assessed.

Materials and Methods: 208 children with organoleptic test (OLT) scores of 2 or more were randomly assigned to four groups: A: conventional oral hygiene practices (COH) including toothbrushing and flossing; B: COH + tongue scraping (TS); C: COH + TS + chlorhexidine; D: COH + TS + chlorhexidine + probiotics. OLT was performed at 1-week and 3-month follow-ups.

Results: A significant and stable number of participants showed major and moderate levels of improvement in OLT scores in group D ($p < 0.001$). The improvement of OLT scores in group C was also significant ($p < 0.001$), but not stable over the follow-ups ($p = 0.44$). Neither significant nor stable improvements in the OLT scores were detected in groups A and B through follow-ups ($p > 0.05$).

Conclusion: Probiotic therapy following oral disinfection with chlorhexidine may reduce the severity of halitosis over longer periods.

Key words: antimicrobial agent, chlorhexidine, halitosis, oral hygiene, probiotics

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Halitosis or oral malodor is any unpleasant odor emerging from the mouth that is detected by others. It may be a result of several intra- and extra-oral factors.[26] Although halitosis has a multifactorial aetiology, localised factors play a major role in most cases; 90% of oral odor originates from the oral cavity as a result of microbial metabolism and the imbalance of the normal microflora of the tongue dorsum, saliva and the periodontal pockets.[2,7]

Several strategies have been developed for either elimination or alleviation of halitosis, targeting the potential origins such as poor oral hygiene, gingival inflammation, dental plaque, dental caries or salivary flow reduction.[2,21] Nonetheless, the cur-

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rent trend focuses on non-selective anti-bacterial treatment to reduce the total number of oral microflora. Such protocols typically require physical or chemical therapy to be carried out daily and only provide a short-term benefit, as the malodor-causing bacteria quickly recover once treatment stops.[10]

Probiotics, by a generally-accepted definition, consist of a live microbial food supplement which beneficially affects the host by improving its intestinal microbial balance. Probiotics confer a health benefit on the host and may have beneficial applications in the reduction of halitosis.[13] The use of probiotics in treatment of gingivitis, periodontal disease[17] and risk reduction of candidal mucosal infections has been described.[23] It has also been proposed that probiotics may have anti-cariogenic activity by inhibition of mutans streptococci.[12] Their mechanism of action is based on their ability to compete with pathogenic microorganisms for adhesion sites such as biofilm or dental plaque and to antagonise these pathogens.[10,28]

Several studies were performed to replace bacteria responsible for halitosis with probiotic bacteria such as *Streptococcus salivarius* (K12), *Lactobacillus salivarius* or *Weissella cibaria*. The general objective is to prevent re-establishment of undesirable bacteria and thereby prevent the reoccurrence of oral malodor.[15,17] A study on individuals with halitosis reported reduced levels of volatile sulphur compounds after consumption of gum or lozenges containing S. *salivarius* (K12).[10] Kang et al[19] showed that *W. cibaria* produces hydrogen peroxide, which inhibits the growth of *Fusobacterium nucleatum* and causes a marked reduction in the production of hydrogen sulphide and methanethiol, hence diminishing foul odors.[19] However, recurrence of oral malodor over a prolonged period persists as a main concern using these protocols.

When levels of oral microbiota are sufficiently diminished in the oral cavity, adding beneficial live bacteria can lead to the switching of a pathogenic condition to a more stable colonisation with probiotic strains.[4] Therefore, it seems reasonable to presume that a marked alteration in the balance between oral microbiota responsible for halitosis and probiotic strains after exposure to an antimicrobial agent (e.g. chlorhexidine) provides a more efficient colonisation of probiotic strains and therefore more long-lasting treatment of oral malodor. Chlorhexidine (CHX) is widely used for chemical plaque control because of its antibacterial effect on both Gram-positive and Gram-negative microorganisms.

The current study was designed to assess the effects of prophylactic use of CHX and subsequent probiotic consumption on oral malodor in children. The study design and hypothesis were based on the principle of competitive exclusion, which favours the beneficial bacteria if they adhere to various parts of mouth before pathogenic strains do so. Thus, the current study compared the effect of probiotic strains along with different mechanical and chemical methods of oral hygiene practice on oral malodor. Four sets of variables, including (a) DMFT/dmft, (b) mechanical and chemical oral hygiene practices, (c) mechanical and chemical oral hygiene practices along with consumption of *S. salivarius* probiotics, (d) oral malodor, in addition to parental satisfaction about the treatment results, were analysed to determine how mechanical/chemical oral hygiene practices along with application of probiotic bacteria influence oral malodor in children to test the hypothesis that a marked alteration in the balance between oral microbiota and probiotic strains – along with routine mechanical methods – can lead to the rapid purging of inherent pathogenic bacterial populations, thereby quickly switching to a more persistent colonisation with probiotic strains and consequently treatment of oral malodor.

MATERIALS AND METHODS

Participants

This randomised clinical trial was performed at the Department of Paediatric Dentistry, Tabriz University of Medical Sciences, during the period from July to October 2014. The children admitted to this department are mostly referrals from general dental practitioners working in the area, and also from Tabriz Paediatric Hospital, for comprehensive assessments as well as routine dental treatments. Once admitted, a comprehensive medical and dental history is taken and a treatment plan is established for each patient.

During the study period, 312 children whose parents reported degrees of oral malodor were selected consecutively through careful screening for the following criteria:

- Children with non-compromised oral health, no clinical signs of gingivitis or periodontal disease and no current orthodontic therapy.
- Absence of systemic conditions or developmental disturbances that have an association with oral malodor, such as diabetes mellitus, renal

disease, gastrointestinal tract disorders, respiratory disease, chronic sinusitis and local/systemic conditions affecting saliva quality and quantity.
- No use of medications affecting the quality and quantity of saliva for the last six months.
- No habitual mouth breathing.
- No previous consumption of probiotic products.

The study design, which was in accordance with the Helsinki Declaration of Human Rights, was submitted to and approved by the Committee for Ethics in Research on Humans at Tabriz University of Medical Sciences (Ref. No.: 7663).

Sample size and power calculation

To estimate sample size based on a mean comparison test, the SAS 9.1 statistical software package (SAS Institute; Cary, NC, USA) was used. According to a pilot study, assuming the mean change in halitosis in the four groups and considering $\alpha = 0.05$ and power = 80%, the 15% outcome difference led to a required sample size of 38 for each group, which was increased to 52 to improve the power of the study and compensate for probable loss to follow-up.

Subject recruitment

At the baseline visit, the objectives of the study and its methodology were explained to the parents. A detailed medical history interview was conducted with the parents, and they were asked to inform the interviewer of any need for antibiotics during the study course. Sample selection consisted of two consecutive phases. In the first phase, a total of 312 healthy children aged 6 to 9 years (182 females and 130 males) with reported oral malodor were enrolled at the Department of Paediatric Dentistry. The malodor was determined using an organoleptic test (OLT), a method used for the direct sniffing of expelled mouth air. The OLT scores were estimated on a 6-point scale of 0 to 5. Subjects having an organoleptic score of 2 or more were recruited. Thereafter, all dental treatments including pulp treatments, restorations or extraction were performed as indicated.

In the second phase, 208 subjects who received dental treatment as needed and had an OLT score of 2 or higher were included in the study.

Randomisation and blinding

A random allocation list was generated using randomisation software (RandList version 1.2, DatIng; Tübingen, Germany) to allocate subjects to each group, one by one according to their order of admission. The operator was not blinded to the interventions because of different manipulation techniques implemented for the studied groups. All other contributors to the study were blinded to the generation and implementation of the treatment assignment.

Study groups and interventions

One week after the first stage, 208 children who had an OLT score of 2 or more were randomly assigned to four groups, each consisting of 52 participants: group A, conventional oral hygiene practices (COH) including toothbrushing and flossing; group B, COH + tongue scraping (TS); group C, chlorhexidine (CHX) + COH+ TS; group D, CHX + COH + TS + probiotics (PB). The parents and children were informed of the nature of the interventions.

All subjects in group A performed COH using toothpaste (Colgate Total, Colgate; Sydney, Australia) and flossing. In group B, COH was followed by TS using a plastic tongue scraper for 30 s. In group C, COH and TS were supplemented by a 30-s rinse with 5 ml of mouthwash containing 0.12% CHX (Peridex, Omni Oral Pharmaceutical; West Palm Beach, FL, USA) twice daily 1 h after toothbrushing for two weeks. The children in group D received COH, TS and CHX mouthwash as described for group C, and then were asked to suck a probiotic lozenge containing >1 x 10^9 colony forming units (CFU) of *S. salivarius* (K12). No toothbrushing was allowed for at least 1 h after lozenge consumption for 2 weeks. As the substantivity of CHX is 8–12 h,[4] consumption of lozenges started 24 h after cessation of CHX application. The subjects attended the Department of Paediatric Dentistry every week for check-ups, and their records were registered in the chart. Those who did not follow the given instructions were excluded from the study during the study course.

Oral malodor assessment

The organoleptic test (OLT) is the simplest method of oral malodor measurement by human judges and reflects an everyday situation. It is considered to be the gold standard for measuring bad breath.[29]

Table 1 Definition of different levels in the organoleptic test (OLT)	
Score	**Description**
0	Absence of odor
1	Questionable odor: odor is detectable, although the examiner could not recognise it as malodor
2	Slight malodor: odor is deemed to exceed the threshold of malodor recognition
3	Moderate malodor: malodor is definitely detected
4	Strong malodor: malodor is objectionable but examiner can tolerate it
5	Severe malodor: overwhelming malodor, examiner cannot tolerate it

The subjects were asked to refrain from eating, drinking, chewing gum, smoking, brushing or rinsing the mouth for at least 5 h. Each subject was instructed to remain quiet with lips closed for a period of 30 s and then asked to exhale through the mouth with a moderate force at a distance of approximately 10 cm from the investigator. The oral malodor scores were recorded on a 6-point scale of 0 to 5 as follows: 0, no odor; 1, barely noticeable odor; 2, slight but clearly noticeable odor; 3, moderate odor; 4, strong odor; 5, extremely foul odor. A score of 2 was diagnosed as halitosis (Tables 1 and 2).

The two examiners were experienced dental hygienists with previous experience in assessment of malodor and no history of chronic allergies or asthma. They had completed a sensory training exercise using smelling test strips dipped in 0-, 10-, 50-, 100-, 500- and 1000-ppb methyl mercaptan (provided by the Faculty of Pharmacy) and conducted pre- and post-training odor measurements during the pilot study. The percentage of agreement in scores exceeded 80% ($\kappa = 0.86$).

On the examination day, to enable the most reliable measurement results, the examiners were asked to restrict their consumption of beverages such as coffee, tea, and juice and avoid smoking and use of scented cosmetics before the OLT measurements. To avoid adaptation of the examiners' sense of smell to possibly detectable odors, there was a break of 5 min after each examination.

OLT measurements were carried out at 1 week and 3 months following interventions by the two examiners who were blinded to the interventions. In case of disagreement, examination by a third examiner was recorded as the outcome. The flow of participants and interventions were followed from allocation to the final data analysis after 3 months (Fig 1).

Parental satisfaction about the treatment result was assessed through parental response to the question 'Are you satisfied with the overall improvement in your child's halitosis?' Parents were asked to rate their responses on a 3-point Likert scale: 1 (not at all satisfied); 2 (moderately satisfied); 3 (completely satisfied).

Table 2 Numerical scale indicating degree of improvement of halitosis in adolescents	
Improvement	**Decrease in degree of halitosis**
Major	Organoleptic score change > 2
Moderate	Organoleptic score change = 2
Slight	Organoleptic score change = 1
No improvement	Organoleptic score change = 0

Statistical analysis

The main statistical tests addressing the research question were the chi-squared test or Fisher's Exact test to assess differences between gender and study groups, and one-way ANOVA to compare quantitative data. A post-hoc test was applied to compare differences between groups. In case of statistical significance, Bonferroni-Holm correction was carried out. Data were analysed using SPSS software (version 16), with $p < 0.05$ considered statistically significant. The Kappa statistic was calculated for interexaminer reliability assessment.

RESULTS

The 208 participants consisted 91 females and 117 males, 6–9 years of age (mean: 7.46 ± 1.09 years), organised into four groups. Eleven subjects were excluded from the study because they did not

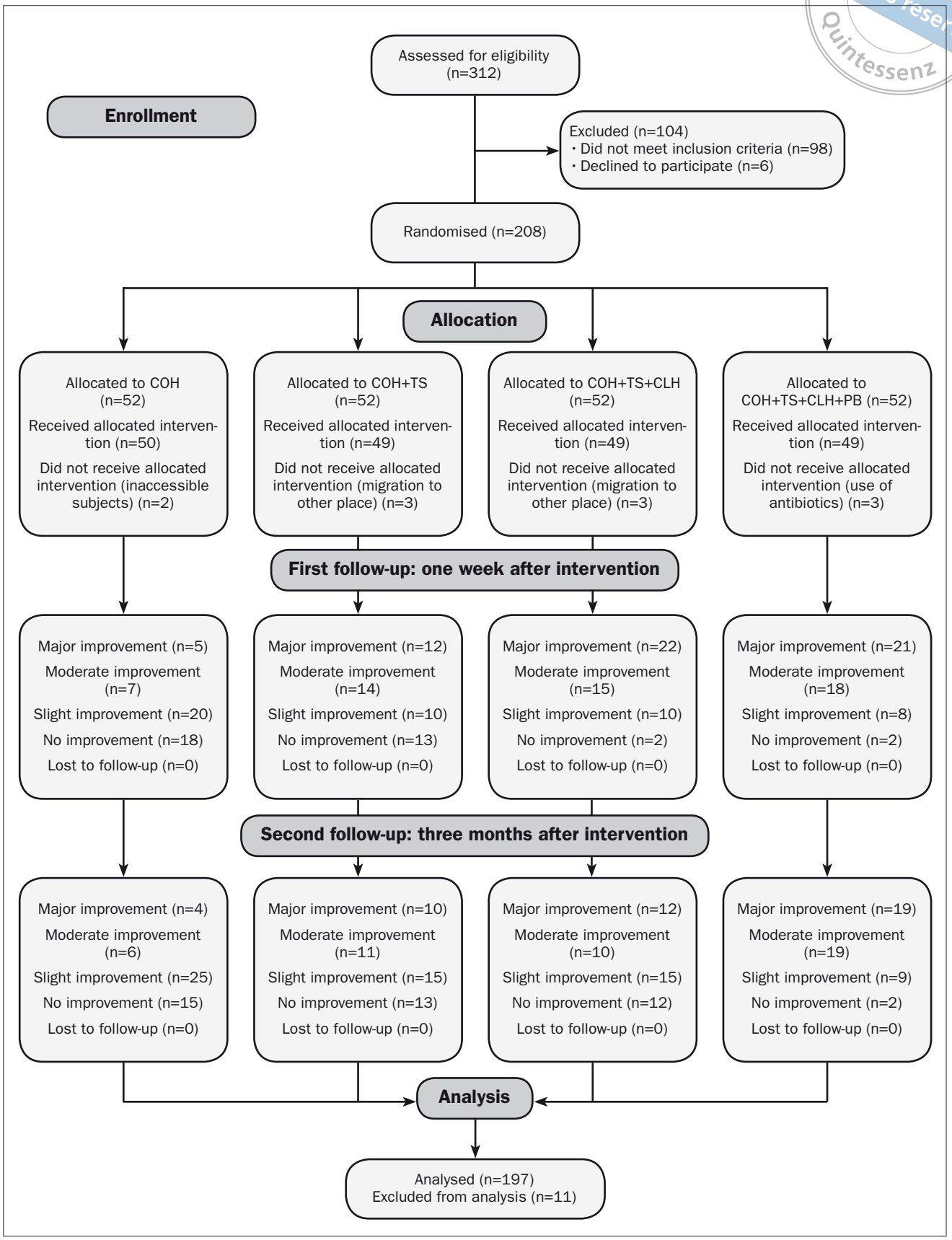

Fig 1 Flow diagramme of study participants.

Table 3 Baseline characteristics of the study samples						
		Group A (n = 50)	Group B (n = 49)	Group C (n = 49)	Group D (n = 49)	p-value
Demographic parameters						
Age (y)		7.54 ± 1.18	7.31 ± 1.12	7.53 ± 1.01	7.45 ± 1.10	0.42*
Sex	Male	27 (54%)	18 (36.7%)	21 (42.9%)	25 (51%)	0.3**
	Female	23 (46%)	31 (63.3%)	28 (57.1%)	24 (49%)	
Clinical parameters						
Number of teeth		24.32 ± 4.08	24.11 ± 4.23	25.68 ± 4.32	25.27 ± 5.76	0.21*
Plaque index		55.22 ± 10.23	52.46 ± 9.35	61.38 ± 8.59	54.73 ± 8.42	0.09*
OLT score		3.02 ± 0.83	2.84 ± 0.72	3.28 ± 0.68	3.15 ± 0.41	0.08*

* Results of one-way ANOVA. ** Results of chi-squared test.

Table 4 Organoleptic test (OLT) score according to the subjects' caries experience				
	Caries experience level			
	Caries free dmft/ DMFT = 0	Low dmft/ DMFT = 1	Moderate 2 ≤ dmft/ DMFT ≤ 3	High dmft/ DMFT ≥ 4	p-value
OLT score	2.6 ± 0.4	2.8 ± 0.5	3 ± 0.3	3 ± 0.2	0.24

complete the trial period. Baseline characteristics of the study samples are shown in Table 3.

The agreement between the two examiners at baseline and the 3-month follow-up was excellent (baseline $\kappa = 0.87$, $p < 0.001$; final follow-up $\kappa = 0.91$, $p < 0.001$). There was no need for a third examiner to make a reference decision.

An increase in the OLT scores was seen with increasing DMFT/dmft levels, but this was not statistically significant ($p > 0.05$; Table 4).

Intragroup results

Group A: A statistically significant number of subjects showed no or slight improvement in OLT scores at the first and second follow-up sessions ($p = 0.03$). Group B: No statistically significant difference was registered between degree of the improvement in OLT scores at the first and second follow-up sessions ($p = 0.18$). Group C: A statistically significant number of the participants had major and moderate levels of improvement in OLT scores at the first follow-up ($p < 0.001$), while the improvement was not statistically significant at the second follow-up ($p = 0.44$). Group D: At the first and second follow-up sessions, a statistically significant number of participants showed major and moderate levels of improvement in OLT scores ($p < 0.001$).

Intergroup comparison

The levels of improvement at the first (F1) and second follow-up (F2) sessions for all groups are shown in Table 5. There was no significant difference in the OLT scores between groups A and B ($p = 0.11$) nor between groups C and D at the first follow-up ($p = 0.27$). However, OLT scores differed significantly between groups B and C ($p = 0.03$), A and C ($p < 0.001$), A and D ($p < 0.001$), and B and D ($p < 0.001$). At the second follow-up, there was no significant difference between groups A and B ($p = 0.51$), groups B and C ($p = 0.18$) or groups A and C ($p = 0.33$). Comparing all groups at the second follow-up, a significant difference in the OLT scores for groups C and D ($p < 0.001$), groups A and D ($p = 0.02$) and groups B and D ($p < 0.001$) was observed. The differences between groups A and C, A and D, and B and D at the first follow-up, and groups B and D, as well as C and D at the second follow-up were still significant after Bonferroni-Holm correction. The improvement of OLT scores in group D was maintained through the first and second follow-ups, while a worsening in other groups was recorded at the 3-month follow-up.

Considering parents' satisfaction with the treatment outcome at the 3-month follow-up session, 39 (79.59%) and 45 (91.83%) parents in group C and D, respectively, were very satisfied regarding

Table 5 Improvements in organoleptic test score in the first and second follow-ups across all study groups

Level of improvement	Group A (N=50)		Group B (N=49)		Group C (N=49)		Group D (N=49)	
	F1	F2	F1	F2	F1	F2	F1	F2
Major	5 (10.0)	4 (8.0)	12 (24.5)	10 (20.5)	22 (44.9)	12 (24.5)	21 (42.9)	19 (38.8)
Moderate	7 (14.0)	6 (12.0)	14 (28.6)	11 (22.4)	15 (30.6)	10 (20.5)	18 (36.8)	19 (38.8)
Slight	20 (40.0)	25 (50.0)	10 (20.4)	15 (30.6)	10 (20.5)	15 (30.6)	8 (16.3)	9 (18.4)
No improvement	18 (36.0)	15 (30.0)	13 (26.5)	13 (26.5)	2 (4.0)	12 (24.4)	2 (4.0)	2 (4.0)
A and B p-values: p F1 = 0.11, p F2 = 0.51								
B and C p-values: p F1 = 0.03, p F2 = 0.18								
C and D p-values: p F1 = 0.27, p F2 < 0.001*								
A and C p-values: p F1 < 0.001*, p F2 = 0.33								
A and D p-values: p F1 < 0.001*, p F2 = 0.02								
B and D p-values: p F1 < 0.001*, p F2 < 0.001*								
* Significant after Bonferroni-Holm correction. F1: first follow-up session; F2: second follow-up session.								

their children's halitosis, while in groups A and B only 21 (42.00%) and 25 (51.02%), respectively, were very satisfied. The difference in parental satisfaction values between groups C and D and groups A and B was statistically significant (p = 0.01).

DISCUSSION

The main objective of the present study was to compare the efficacy of mechanical methods of oral hygiene practice with and without CHX and probiotic bacteria (*S. salivarius*) on oral malodor.

S. salivarius appears to have excellent credentials as an oral probiotic because it is unlikely to contribute significantly to oral malodour.[31] It has been shown that *S. salivarius* (K12) suppressed the growth of the various reference strains of bacteria implicated in halitosis. More importantly, this bacterial strain is a pioneer coloniser of oral surfaces and is a numerically predominant nondisease-associated member of the oral microbiota of healthy humans.[20] It is also is known as the commensal probiotic of the oral cavity because of its ability to produce bacteriocins that contribute to the reduction of bacterial species implicated in halitosis. In vitro testing has shown that *S. salivarius* K12 suppresses the growth of black-pigmented bacteria in the oral cavity. Reduced levels of volatile sulphur compounds after consumption of gum or lozenges containing *S. salivarius* K12 have been reported in patients with halitosis.[20]

We found the chemical method of oral hygiene practice using CHX in combination with conventional methods to induce a significant decrease in oral malodor in groups C and D compared to that in groups A and B. Considering the bacterial activity of mainly gram-negative anaerobes as the major origin of persistent oral malodor,[3] it is a plausible postulate that improvement of oral hygiene along with the use of antimicrobial products can often prevent or manage oral malodor due to a decrease in bacterial activity and population.[25] Similarly, current evidence shows good short-term reduction in halitosis scores with CHX, while long-term use is neither recommended nor effective.[8] CHX is considered the gold standard and primary agent for controlling plaque and gingivitis.[20] The antibacterial activity of CHX is related to the cationic molecule, which is rapidly attracted by the negatively charged bacterial cell surfaces. After adsorption, the integrity of the bacterial cell membrane is altered, which results in a reversible leakage of bacterial low molecular-weight components at low dosage or more severe membrane damage at higher doses.[27] However, long-term use of CHX is associated with extrinsic staining of the teeth and tongue, increased calculus formation, irritation of oral mucosa, burning sensation and alteration of the taste sense.[30]

Although the chemical method of oral hygiene practice in group C yielded a significant decrease in halitosis score at the first follow-up session, the reoccurrence of oral malodor at the second follow-up confirmed the main concern of previous studies on reducing oral malodor using CHX. Conversely, improved halitosis values in group D did not show significant differences between the follow-ups. Therefore, the result seen in group D confirms our

hypothesis that probiotic therapy following oral disinfection may be an effective approach for longer-term control of oral malodor. The principle of competitive exclusion, meaning the suppression of oral pathogens using CHX and promotion of the growth of probiotics, seems to provide a reasonable explanation for this finding. In fact, based on the principle of competitive exclusion, a reduction in oral microbial counts provides a unique opportunity for easy colonisation with probiotic strains.[5,29] Accordingly, effective biofilm control strategies should control microbial activity to prevent colonisation with selected microorganisms while supporting the growth of other selected species.[5,29] Our results are in agreement with those of Iwamoto et al,[18] who showed that oral administration of probiotic lactobacilli primarily improved oral halitosis. However, considering the small number of participants and the short follow-up period, they suggested further work to improve the stability of *L. salivarius* in the complex oral microflora and allow it to maintain its activity.[24]

The results obtained in groups A and B are not surprising. Relative improvement in group B compared to that in group A may be attributed to the fact that oral malodor arises mainly from the resident microbes – particularly anaerobic ecosystems – on the dorsum of the tongue. The papillary structure of the dorsum represents a unique ecological niche in the oral cavity, offering a large surface area that favors the accumulation of oral debris and microorganisms. Therefore, the posterior dorsum of the tongue is the principal site for the bacterial mass producing malodorous compounds.[16,24] It has been suggested that there is a direct relationship between the quantity of microbes present and the degree of odor.[16]

Although the result of the preliminary phase of the study revealed a relationship between DMFT/dmft and halitosis, OLT scores according to different levels of the DMFT/dmft did not differ significantly. A small number of studies investigating the relationship between caries and halitosis have shown inconsistencies in their findings. Evirgen et al[14] suggested there is no significant relationship between DMFT/dmft and halitosis. It is reported that young children with oral malodor are caries free; whereas age-matched children without malodor have moderate to high caries activity.[22] The contradictory results may be related to the fact that the aetiological factors of caries, periodontal diseases and oral malodor are mostly associated with bacterial accumulation and plaque composition. Some of the bacteria causing caries, such as *Lactobacillus* sp. and *Porphyromonas* sp., have also received considerable attention as pathogens responsible for halitosis. In addition, recent findings have shown that an increase in the salivary pH and buffering capacity and decrease in viscosity is linearly related to the number of eliminated carious tooth surfaces.[7,10] Therefore, it seems logical to assume that lower carbohydrate accumulation and salivary viscosity, plaque removal, increased pH and buffering capacity result in the elimination of cariogenic bacteria and caries, and finally less halitosis.[7]

Since providing quality outcomes as well as patient satisfaction with treatment is the priority and the primary competitive edge in any healthcare system, we also evaluated the extent to which the parents' satisfaction with the improvement in halitosis differed from that of the healthcare practitioners. Interestingly, the perceptions of parents and practitioners were consistent regarding the level of improvement in halitosis. Current evidence reveals that patient satisfaction surveys using ratings are leading indicators of healthcare outcomes, including compliance with medical advice, likelihood to recommend, and return visits for care.[11,24] A patient-centered approach should take into account the patient's needs, expectations, and evaluations for the purpose of quality improvement. It is important to help parents and their children see the situation during treatment planning the same way dentists sees it, so that everyone has similar expectations of the treatment.

CONCLUSIONS

Based on the results of the present study, it can be concluded that mechanical removal of biofilm and microorganisms responsible for oral malodor is the first step in controlling halitosis.[9] In view of the fact that 60% of halitosis originates from the surface of the tongue,[24] it is reasonable to recommend tongue scraping as a main mechanical oral hygiene practice to prevent halitosis. In addition, the mechanical oral hygiene methods could be supplemented by a chemical regimen (e.g. CHX) as a superior approach in removing biofilm.[6] According to the present results, probiotic therapy following oral disinfection with CHX may be a practical method to induce a persistent switch in the oral microbiota and improve malodor.

However, a generalisation of the present findings to broader implementation necessitates further investigation, particularly considering selected oral

biofilm and microbial activities to prevent colonisation of selected organisms while supporting the growth of probiotic bacteria to establish control strategies. Further studies, including large-scale randomised clinical trials, are needed to determine the efficacy of other probiotic strains targeting microorganisms responsible for oral malodor, as well as other salivary parameters.

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