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ORIGINAL ARTICLE

The short-term treatment effects on the microbiota at the dorsum of the tongue in intra-oral halitosis patients—a randomized clinical trial

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Abstract
Objectives This study aims to assess the effects of rinsing with zinc- and chlorhexidine-containing mouth rinse with or without adjunct tongue scraping on volatile sulfur compounds (VSCs) in breath air, and the microbiota at the dorsum of the tongue.
Material and methods A randomized single-masked controlled clinical trial with a cross-over study design over 14 days including 21 subjects was performed. Bacterial samples from the dorsum of the tongue were assayed by checkerboard DNA–DNA hybridization.
Results No halitosis (identified by VSC assessments) at day 14 was identified in 12/21 subjects with active rinse alone, in 10/21with adjunct use of tongue scraper, in 1/21 for negative control rinse alone, and in 3/21 in the control and tongue scraping sequence. At day 14, significantly lower counts were identified only in the active rinse sequence ($p<0.001$) for 15/78 species including, *Fusobacterium* sp., *Porphyromonas gingivalis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Tannerella forsythia*. A decrease in bacteria from baseline to day 14 was found in successfully treated subjects for 9/74 species including: *P. gingivalis*, *Prevotella melaninogenica*, *S. aureus*, and *Treponema denticola*. Baseline VSC scores were correlated with several bacterial species. The use of a tongue scraper combined with active rinse did not change the levels of VSC compared to rinsing alone.
Conclusions VSC scores were not associated with bacterial counts in samples taken from the dorsum of the tongue. The active rinse alone containing zinc and chlorhexidine had effects on intra-oral halitosis and reduced bacterial counts of species associated with malodor. Tongue scraping provided no beneficial effects on the microbiota studied.

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Clinical relevance Periodontally healthy subjects with intra-oral halitosis benefit from daily rinsing with zinc- and chlorhexidine-containing mouth rinse.

Keywords Bacteria · Halitosis · Mouth rinse · Tongue scraping · VSC

Introduction

Intra-oral halitosis is a social and psychological problem that is prevalent among adults [1–3]. Predominantly, the presence of hydrogen sulfide (H_2S), methyl mercaptan (MM), in exhaled air has been associated with intra-oral halitosis [4, 5]. Levels of volatile sulfur compounds (VSC) can be measured by gas chromatography [6, 7] or by a device that accounts for sulfuric gases in general (total volatile sulfur compounds, T-VSC). There appears to be a poor correlation between self-reported perception of intra-oral halitosis and VSC measurements of intra-oral halitosis [3]. Organoleptic scoring (OLS) which includes the assessment of breath air by a trained examiner is considered as the gold standard for the diagnosis of intra-oral halitosis [8].

Through the degradation of sulfur-containing amino acids, several bacterial species produce VSCs in periodontal pockets and on the tongue [9–13]. In the presence of cysteine and methionine, probiotic bacteria such as *Lactobacillus acidophilus*, *Lactobacillus plantarum*, *Lactobacillus rhamnosus*, and *Lactobacillus reuteri* may also generate considerable amounts of VSCs [14].

Microbiological data suggest that the levels of *Treponema denticola* and *Fusobacterium nucleatum* in bacterial samples taken from the dorsum of the tongue, and from saliva can be associated with intra-oral halitosis [15, 16]. Studies have shown that the greatest number of bacteria associated with intra-oral halitosis can be found at the dorsal part and posterior to the circumvallate papillae of the tongue [17].

Intra-oral halitosis in children has been identified with elevated levels of *Prevotella intermedia* in supragingival dental plaque [18]. Oral counts of *Prevotella melaninogenica* have been associated with intra-oral halitosis in older subjects [19]. In nonsmokers with intra-oral halitosis, a high prevalence of Enterobacteriaceae and Pseudomonadaceae on the dorsum of the tongue has been observed in subjects>40 years [20]. *Helicobacter pylori* produces H_2S and MM, suggesting that this microorganism may contribute to the development of intra-oral halitosis [21, 22]. A greater bacterial diversity can be observed in subjects with intra-oral halitosis than in subjects without intra-oral halitosis [23]. Intra-oral halitosis may also be the result of complex bacterial interactions including presently many uncultivable bacteria [24].

Periodontitis in combination with tongue coating has been considered as a primary factor for intra-oral halitosis (1). Different treatment strategies have been proposed to manage intra-oral halitosis. This includes mechanical debridement of periodontal pockets, and rinsing with antimicrobial agents and/or metal salts [25, 26]. Several different mouth rinses are today proposed to reduce intra-oral halitosis [27–29]. The use of a dentifrice containing sodium lauryl sulfate may prevent VSC formation in morning intra-oral halitosis in periodontally healthy subjects [30]. A zinc containing dentifrice may also reduce intra-oral halitosis [31]. Other studies have shown that periodontal treatment using Nd/Yag lasers may reduce the amount of intra-oral halitosis [32]. In addition, tongue scraping in combination with periodontal therapy may reduce VSCs in breath air [33]. This change in VSC may not be associated with changes in bacterial counts [34, 35]. In fact, the effect of tongue scraping is transient and of very short duration [35]. Others have shown that tongue scraping reduces the counts of bacteria associated with intra-oral halitosis, and specifically related to the counts of *Porphyromonas gingivalis* [36].

Study aims

This study aims to investigate in subjects with confirmed intra-oral halitosis but without evidence of periodontitis if (1) the microbiota at the dorsum of the tongue is related to VSC and (2) if any of four different treatment modalities employed over 14 days reduced the counts of individual bacteria at the dorsum of the tongue.

Materials and methods

The Ethics Committee at Lund University, Sweden approved the study. All subjects signed informed consent. Approved advertisements in the local newspaper, message boards, and on the web page of the University of Kristianstad, Sweden were used to recruit study subjects. The study was performed between 2008 and 2009 at the dental clinics of the University of Kristianstad. The following criteria were followed to enroll study subjects at the screening visit:

Inclusion criteria: (1) halitosis of intra-oral origin, (2) OLS ≥2, (3) a level of TVSC≥160 parts per billion (ppb) determined with a device (Halimeter® Interscan Corporation Chatsworth, CA, USA) assessing T-VSC.

Exclusion criteria: (1) untreated periodontitis defined as having periodontal pockets with a probing pocket depth≥6 mm, (2) open caries lesions, (3) pregnancy, (4) systemic medication related to xerostomia, (5) systemic antibiotic therapy within the preceding 3 months, (6) current smoker, and (7) gastro-esophageal reflux. Bleeding index was used to assess the extent of gingivitis. Gingival inflammation was not used as exclusion criteria.

A medical history and analysis of exhaled air from the nose was assessed to rule out halitosis by other origin than oral halitosis (intra-oral halitosis). An organoleptic score was used to define intra-oral halitosis [36]. Subjects were given written instruction regarding food intakes and oral hygiene.

The subjects were instructed (1) not to consume food containing onions, garlic, or hot spices 48 h before assessments; (2) not to drink alcoholic beverages during the preceding 12 h; (3) not to eat or drink 5 h before assessments (but were allowed to drink water until 3 h before examination); (4) not to perform oral hygiene measures, tongue cleaning, or the use of mouth rinses the morning of the examination; and (5) not use scented cosmetics or aftershave lotions the same morning as the study examinations were performed. The study subjects came to the laboratory for measurements in the morning at day 1; and at day 14, 8–12 h after the last intervention the evening before. At these time points, assessments of VSC were made, and bacterial samples were taken from the dorsum of the tongue.

The following four test periods were performed: (1) active rinse alone, (2) active rinse with the use of a tongue scraper, (3) negative control rinse alone, (4) negative control rinse with the use of the tongue scraper. Each sequence lasted for 2 weeks and was separated by a washout period of 1 week. Subjects were randomized to different order of use for the four study protocols by a computer-based randomization program (IBM® SPSS® Statistics Standard 17.0 software package for PC, IBM Corp. Somers, NY, USA).

The following two solutions were distributed in coded bottles. The active mouth rinse (SB12®, Antula Healthcare AB, Stockholm, Sweden) contained water, glycerine, sorbitol, alcohol (1.8 %), zinc acetate (0.3 %), chlorhexidine diacetate (0.025 %), sodium fluoride (0.05 %), hydrogenated castor oil, citric acid, potassium acesulfame, menthol, and mentha piperita. The negative control rinse solution contained the same flavoring agent (menthol) but without zinc acetate (0.3 %), chlorhexidine diacetate (0.025 %), or sodium fluoride (0.05 %). According to the study protocol, a tongue scraper (Halita®, DentAid, Barcelona, Spain) was also used in two of the study sequences.

The subjects were instructed to rinse with 10 ml of the provided solution during 1 min twice daily and then to spit out the rinse solution. The subjects were instructed to rinse after breakfast and before bedtime. For the adjunct use of the tongue scraper, the subjects were instructed and trained in how to use the tongue scraper. Briefly, they were shown to pull out the tongue, apply the tongue scraper to the dorsum of the tongue and perform five strokes. They were instructed to reach as far posterior as possible at the dorsum of the tongue. This procedure was to be performed twice daily and before using the rinsing solution.

Clinical parameters

Measurements of H_2S, MM, and dimethyl sulfide (DMS) levels were performed with a portable gas chromatograph (OralChroma™, Abilit Corp, Osaka, Japan) and by a device assessing the total VSC (Halimeter® Interscan Corporation Chatsworth, CA, USA). Subjects were defined as being effectively treated for intra-oral halitosis if the VSC values were below specific cut of levels as studied by others (eight). If the subject had T-VSC values$<$160 ppb and a H_2S value$<$112 ppb, and a MM value$<$26 ppb following therapy, treatment was considered as successful.

Microbiological processing

Bacterial samples were taken with Catch all swabs (Catch-All™, Sample collection swab, Epicentre, Madison, WI, USA). The swab was moved across the dorsum of the tongue in several strokes back to forward as well as across the tongue. Efforts were made to rotate the swab and to include the full extent of the dorsum of the tongue to the extent possible. The swab was then placed in a transport vial designed for such swab samples. Vials were labeled with subject-specific identification and time point of sampling. To each tube with tongue bacterial samples, 300 μl Tris EDTA buffer (10 mM Tris–HCl, 1.0 mM EDTA at pH 7.6) was added. After 10 min, these samples were sonicated during 10 s. Subsequently, 200 μl of freshly made 0.5 M NaOH was added to each vial and the swab was removed. The samples were processed by checkerboard DNA–DNA hybridization as previously described [37–40]. A software program (ImageQuant, Amersham Pharmacia, Piscataway, NJ, USA) was used to analyze the digitized information. Signals were compared against standard lanes of known bacterial amounts (10^5 cells). Signals were converted to absolute counts by comparison with these standards and studied as the proportion of sites defined as having $\geq 1.0\times10^5$ bacterial cells. A total of 74 bacterial species were studied (Table 1). Cross-reactivity was routinely tested in the microbiology laboratory between known pure bacterial standards (Table 1) and consistent with reports elsewhere [40].

Statistics

Statistical analysis by Kolmogorov–Smirnov tests identified that the study data did not present with a normally distribution pattern. Statistical analysis was performed using non-parametric test including Mann–Whitney *U* tests and related samples Wilcoxon signed-rank test to assess differences in bacterial counts between and within study groups. Correlations between bacterial counts and VSC scores were assessed with Pearsons' and Spearman rank bivariate

Table 1 Reference bacterial strains included in the DNA–DNA checkerboard analysis

Species	Collection	Species	Collection
A. israelii	ATCC 12102	*Lactobacillus jensenii*	GUH 160339
A. naeslundii (type I + II)	ATCC 43146	*Lactobacillus vaginalis*	GUH 078092
A. neuii	GUH 550898	*Leptotrichia buccalis*	ATCC14201
A. odontolyticus	ATCC 17929	*Mobiluncis curtisii*	GUH 070927
A. actinomycetemcomitans (a)	ATCC29523	*M. mulieris*	GUH 070926
Aggregatibacter actinomycetemcomitans (Y4)	ATCC 43718	*Neisseria mucosa*	ATCC 33270
A. christensenii	GUH 070938	*P. micra*	ATCC 19696
Aanaerococcus vaginalis	GUH 290486	*Peptoniphilus* sp.	GUH 55097
A. parvulum	GUH 160323	*Porphyromonas endodontalis*	ATCC 35406
Atopobium vaginae	GUH 010535	*P. gingivalis*	ATCC 33277
Bacteroides ureolyticus	GUH 080189	*P. bivia*	GUH 450429
Bifidobacterium biavatii	GUH 071026	*P. disiens*	GUH 190184
Bifidobacterium bifidum	GUH 070962	*P. intermedia*	ATCC 25611
Bifidobacterium breve	GUH 080484	*P. melaninogenica*	ATCC 25845
Bifidobacterioum longum	GUH 180689	*Propionibacterium acnes*	ATCC 11727/2
Campyobacter gracilis	ATCC 33236	*Proteus mirabilis*	GUH 07092
C. rectus	ATCC 33286	*Pseudomonas aeruginosa*	DSMZ 50071
Campylovacter showae	ATCC 51146	*Selenomonas noxia*	ATCC 43541
Capnocytophaga gingivalis	ATCC 33612	*Staphylococcus anaerobius*	DSMZ 20714
Capnocytophaga ochraceae	ATCC 335945	*Staphylococcus aureus*	ATCC 25923
Capnocytophaga sputigena	ASTCC 33612	*Staphylococcus aureus* (yellow)	GUH 070921
Corynebacterium nigricans	GUH450453	*Staphylococcus aureus* (white)	GUH 070922
Cornerbacterium aurimucosum	GUH 071035	*Staphylococcus epidermidis*	GUH 130381
Dialister sp.	GUH 071045	*Staphylococcus haemolyticus*	DSMZ 20263
Escherichia coli	GUH 070903	*Streptococcus agalactiae*	GUH 230282
Eikenella corrodens	ATCC 23834	*Streptococcus anginosus*	ATCC 33397
Enterococcus faecalis	GUH 170812	*S. constellatus*	ATCC 27823
Enterococcus faecalis	ATCC 29212	*Streptococcus gordonii*	ATCC 10558
F. nucleatum nucleatum	ATCC 25586	*Streptococcus intermedius*	ATCC 27335
F. nucleatum polymorphum	ATCC 10953	*S. mitis*	ATCC 49456
Fusobacterium nucleatum naviforme	ATCC 49256	*Streptococcus oralis*	ATCC 35037
Fusobacterium periodonticum	ATCC 33693	*Streptococcus pneumoniae*	DSMZ 11866
Gardnerella vaginalis	GUH 080585	*Streptococcus sanguinis*	ATCC 10556
Haemophilus influenzae	ATCC 49247	*Streptococcus mutans*	ATCC 25175
H. pylori	ATCC 43504	*T. forsythia*	ATCC 43037
L. acidophilus	ATCC 11975	*T. denticola*	ATCC 35405
Lactobacillus crispatus	GUH 160342	*Treponema socranskii*	D40DR2
Lactobacillus gasseri	GUH 17085	*Varibaculum cambriense*	GUH 070917
Lactobacillus iners	GUH 160334	*V. parvula*	ATCC 10790

ATCC Reference strain from the American Type Culture Collection; *D* reference strain from Forsyth Institute, Boston, MA; *GUH* reference strain from Ghent University Hospital Collection, Ghent, Belgium; *DSMZ* reference strain from the German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany

correlation. Due to multiple observations, significance was declared at $p<0.001$. The statistical analysis was performed with a statistical software package (IBM® SPSS® Statistics Standard 18.0 software package for PC, IBM Corp., Somers, NY, USA).

Results

The screening of 53 subjects resulted in the inclusion of 11 females, and 10 males with a diagnosis of intra-oral halitosis. All 21 subjects completed the study. The mean age of

the study subjects was 45.7 years (SD, ±13.3; range, 21–66). Bleeding on probing at ≥20 % of surfaces (four per tooth) was on average found in 23.8 % (5/21) of the subjects, and with the highest subject BOP score at 35 %. Statistical analysis failed to demonstrate baseline differences in all VSCs scores and bacterial counts in samples collected from the tongue dorsum between subjects with a bleeding index below 20 %, or at ≥20 % of surfaces assessed. Statistical analysis also failed to identify significant correlations between the percentage of sites with bleeding and T-VSC, H_2S, MM, and DMS values.

Mean values and SD for T-VSC at baseline and at day 14 are presented for the four study sequences (Table 2). Statistically significant differences between baseline and day 14 were observed for the active rinse and active rinse and tongue scraping sequences ($p<0.01$). At day 14 in the active rinse sequence, 12/21 (57.1 %) subjects were identified by VSC assessments as not having intra-oral halitosis whereas 10/21 (47.6 %) were identified as not having intra-oral halitosis when they participated in the study sequence using active rinse and with the tongue scraper. At day 14 and in the negative-control rinse sequence without tongue scraper 1/21 (4.8 %) and in the control rinse plus tongue scraping sequence, 3/21 (14.3 %) was identified by the VSC assessments as not having a diagnosis of intra-oral halitosis.

At baseline for each sequence, all subjects had an OLS score >2. At day 14, 8/21 (38, 1 %) subjects in the active rinse alone sequence were identified with an OLS ≤1. In the active rinse sequence with tongue scraping, 7/21 (33.3 %) subjects were identified with an OLS ≤1. At day 14 and in the negative control rinse group, 5/21 (23.8 %) subjects were identified with an OLS ≤1. In the negative control rinse and tongue scraping sequence 10/21 (47.6 %), subjects were identified with an OLS ≤1. At day 14, the T-VSC scores were significantly higher in subjects with an OLS ≥2 ($p<0.001$; Fig. 1).

Statistical analysis identified that in the active rinse sequence, the bacterial counts at day 14 were significantly lower in subjects who were effectively treated than in subjects who were identified by the VSC cutoff definition as having intra-oral halitosis also at day 14 for the following species ($p<0.001$): *Aerococcus christensenii*, *Actinomyces israelii*, *Actinomyces naeslundii*, *C. gingivalis*, *Eubacterium saburreum*, *F. nucleatum* sp. *naviforme*, *F. nucleatum* sp. *polymorphum*, *Mobiluncus mulieris*, *Peptostreptococcus anaerobius*, *P. gingivalis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* ATCC, *S. aureus* yellow strain, *S. aureus* white strain, and *Tannerella forsythia*. Statistical analysis failed to demonstrate differences in bacterial counts at day 14 for the other three rinse sequences between subjects who were effectively treated for intra-oral halitosis in comparison to the subjects who were identified by the VSC cutoff definition as having intra-oral halitosis.

Table 2 Distribution of total VSC scores (mean values and standard deviation) at baseline and day 14 for the for study sequences

	Baseline		Day 14		Sign
	Mean	SD	Mean	SD	
Active rinse	262.9	264.6	122.0	55.3	0.01
Control rinse	242.1	215.5	221.7	166.6	NS
Active rinse + tongue scraping	220.9	163.1	130.9	132.1	0.01
Control rinse + tongue scraping	246.1	231.6	193.5	125.5	NS

Within-subject analysis for microbiological changes between pretreatment baseline and at day 14 in the active rinse sequence, for subjects with a successful treatment outcome bacterial counts of the following species decreased ($p<0.001$): *Aggregatibacter actinomycetemcomitans* (Y4), *C. gingivalis*, *Campylobacter rectus*, *F. nucleatum* sp. *naviforme*, *Parvimonas micra*, *P. gingivalis*, *P. melaninogenica*, *S. aureus* ATCC, and *T. denticola*. Statistical analysis failed to demonstrate differences in changes of bacterial load between baseline and day 14 for the three other treatment modalities.

The proportional distributions of selected bacteria defined as being present or absent based on a threshold value definition ($\geq 1.0\times10^5$ bacterial cells) for subjects in the active rinse sequence with or without the use of a tongue scraper are presented (Table 3). Descriptive statistics are provided for these bacteria at day 14 defined by successful or unsuccessful treatment of for subjects in the active rinse sequence without the use of a tongue scraper (Table 4). For all other bacterial species, statistical analysis failed to demonstrate differences at baseline and day 14 between the four study sequences.

The association between bacterial counts and the levels of the three VSCs studied using the baseline data revealed that VSC levels were significantly correlated to the following bacterial species: *A. israelii*, *Actinomyces neuii*, *Actinomyces odontolyticus*, *A. actinomycetemcomitans* (*serotype a*), *Atopobium parvulum*, *Prevotella bivia*, *Prevotella disiens*, *Prevotella nigrescens*, *P. aeruginosa*, *Staphylococcus epidermis*, *Staphylococcus constellatus*, *Streptococcus mitis*, *T. forsythia*, and *Veillonella parvula*. These species were then further studied, but only those present at bacterial counts $\geq 1.0\times10^5$ bacterial cells were included. The distributions of bacterial counts are presented for those bacteria in relation treatment outcomes for group 1 (rinsing with the active ingredient; Fig. 2) and for group 2 (rinsing with active ingredient and the use of a tongue scraper; Fig. 3).

Discussion

Intra-oral halitosis is commonly defined by OLS scoring. Analysis of H_2S, MM, and DMS in exhaled air provides

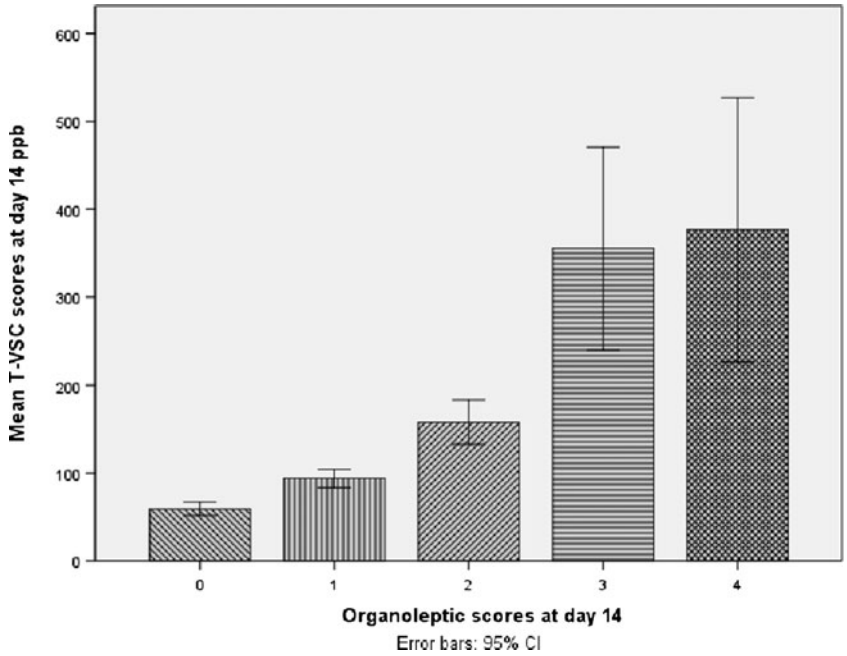

Fig. 1 Mean values and 95 % confidence interval for T-VSC by organoleptic scores (OLS) at day 14 (all sequences included)

valuable information of gases that are associated with intra-oral halitosis. Consistent with other studies, we identified that OLS and VSC scores were correlated [8, 41]. The present data also demonstrated that the impact of study procedure on intra-or halitosis was consistent between OLS and VSC scoring. As pointed out by others, OLS scores may be considered as subjective assessments whereas the VCS analyses with a device allow objective results and

Table 3 Proportional distribution of selected bacteria at baseline and study day 14 presented as the proportion of bacterial counts ≥ threshold value 10^5 bacterial cells in the active rinse sequence with or without tongue scraping

Species	Active rinse alone		Active rinse with tongue scraping	
	Baseline $\geq 10^5$	Day 14 $\geq 10^5$	Baseline $\geq 10^5$	Day 14 $\geq 10^5$
A. israelii	64.0	44.0	66.0	34.0
A. naeslundii	68.0	68.0	64.0	40.0
A. neuii	4.0	4.0	4.0	4.0
A. actinomycetemcomitans (a)	76.0	48.0	64.0	56.0
A. actinomycetemcomitans (Y4)	80.0	60.0	60.0	68.0
B. ureolyticus	4.0	0.0	0.0	4.0
F. nucleatum naviforme	80.0	60.0	72.0	56.0
F. nucleatum nucleatum	80.0	68.0	76.0	72.0
F. nucleatum polymorphum	68.0	52.0	60.0	44.0
F. periodonticum	76.0	68.0	68.0	52.0
H. pylori	40.0	8.0	36.0	66.0
L. acidophilus	84.0	64.0	80.0	66.0
P. micra	52.0	28.0	36.0	48.0
P. gingivalis	36.0	22.0	16.0	8.0
P. endodontalis	0.0	0.0	0.0	0.0
P. bivia	43.0	40.0	28.0	8.0
P. disiens	28.0	20.0	20.0	8.0
P. intermedia	64.0	52.0	64.0	44.0
P. melaninogenica	4.0	96.0	0.0	64.0
P. aeruginosa	0.0	0.0	4.0	0.0
T. forsythia	66.0	48.0	56.0	28.0
T. denticola	4.0	4.0	24.0	12.0

Table 4 Descriptive data for bacteria identified as affected by the active rinse sequence for subjects who at day 14 were defined as not having, or having intra-oral halitosis

Species	Mean	SD	Median	25th percentile	75th percentile
	Halitosis negative subjects at day 14 (n=12)				
A. christensenii	0.14	0.1	0.17	0.10	0.19
A. israelii	0.10	1.36	0.70	0.33	0.85
A. naeslundii	1.39	1.98	0.91	0.63	1.23
C. gingivalis	0.48	0.43	0.31	0.23	0.68
F. nucleatum naviforme	0.88	0.80	1.02	0.68	1.47
F. nucleatum polymorphum	1.13	0.59	0.69	0.58	1.00
M. mulieris	0.13	0.14	0.12	0.00	0.20
P. anaerobius	0.18	0.22	0.18	0.00	0.30
P. gingivalis	0.35	0.31	0.28	0.05	0.59
P.aeruginosa	0.09	0.10	0.07	0.00	0.16
S. aureus	0.18	0.11	0.15	0.12	0.30
S. aureus (yellow)	0.10	0.08	0.09	0.02	0.16
S. aureus (white)	0.09	0.07	0.08	0.04	0.14
S. epidermidis	0.32	0.27	0.27	0.15	0.46
S. haemolyticus	0.24	0.12	0.21	0.15	0.35
T. forsythia	0.81	1.01	0.52	0.08	0.91
	Halitosis positive subjects at day 14 (n=9)				
A. christensenii	0.35	0.16	0.32	0.24	0.44
A. israelii	1.83	0.81	1.74	1.10	2.55
A. naeslundii	1.84	0.69	1.59	1.29	2.46
C. gingivalis	2.96	3.48	1.12	0.64	5.54
F. nucleatum naviforme	2.65	1.16	2.34	2.02	2.97
F. nucleatum polymorphum	3.03	1.94	2.45	1.69	3.94
M. mulieris	0.37	0.30	0.25	0.19	0.47
P. anaerobius	0.62	0.29	0.63	0.36	0.85
P. gingivalis	0.83	0.24	0.81	0.64	1.03
P. aeruginosa	0.27	0.10	0.26	0.18	0.34
S. aureus	0.53	0.16	0.55	0.40	0.65
S. aureus (yellow) strain	0.32	0.11	0.33	0.24	0.40
S. aureus (white) strain	0.27	0.15	0.23	0.17	0.41
S. epidermidis	0.73	0.33	0.74	0.40	0.99
S. haemolyticus	0.55	0.35	0.52	0.26	0.71
T. forsythia	1.61	0.73	1.26	1.02	2.14

Bacterial counts are presented as the proportion of bacterial counts ≥ threshold value 10^5 bacterial cells

with differentiation between different volatile gases (8). In the present study, we used VSC analysis rather than OLS to obtain results that could be associated with VSC-producing bacteria.

The composite VSC outcome score defined by threshold values for VSC assessments was introduced because it remain unclear if it requires one or more of these volatile gases to result in a perception of intra-oral halitosis. One of the problems with VSC data is the lack of a normal distribution pattern and the widespread of scores between subjects. This further supports the use of a combined VSC score.

Data suggest that bacteria associated with periodontitis in subjects with periodontitis may contribute to intra-oral halitosis [26]. The present study design that only included subjects with no evidence of present periodontitis allowed us to assess whether bacteria from the dorsum of the tongue could explain intra-oral halitosis defined by objective assessments of VSCs to exclude the impact on exhaled air as an effect of periodontitis. Previous studies have identified that the dorsum of the tongue can be a primary location for bacteria that produce VSCs [1, 33, 36].

In the present study, the adjunct use of a tongue scraper did not contribute to reduce the number of individuals identified by VSC assessments as not having intra oral halitosis. The study also demonstrated that the use of the tongue scraper failed to alter the microbiota in samples from the dorsum of the tongue. Our results are consistent with

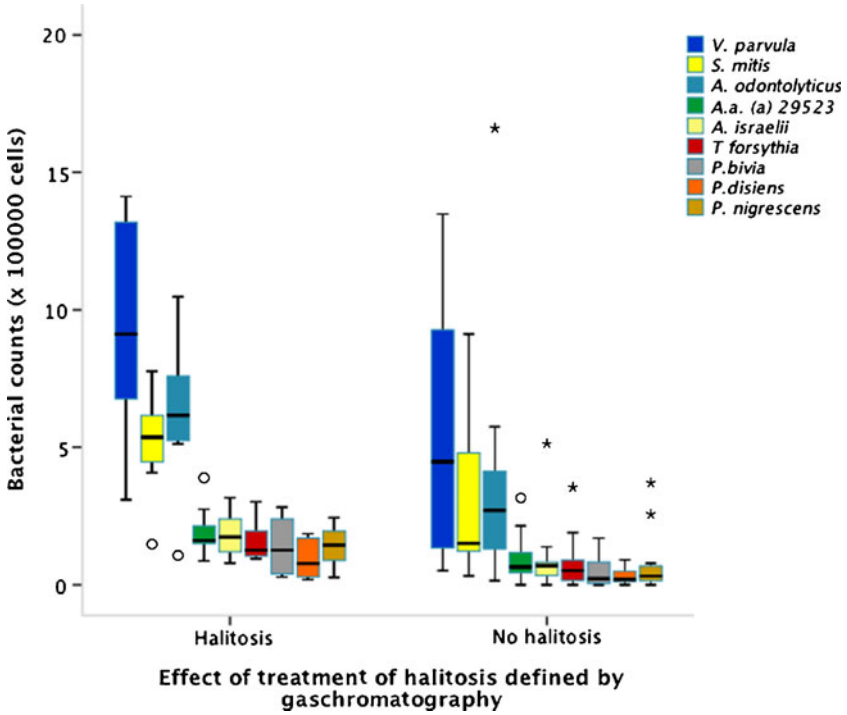

Fig. 2 Boxplot diagram (median values, 25th and 75th percentiles; *empty circle* outlier values, *asterisks* extreme outlier values) presenting bacterial changes in effectively and non-effectively treated subjects in the active rinse sequence

another study also demonstrating that tongue scraping does not reduce bacterial counts at the dorsum of the tongue and that the microbiota at the tongue is resilient [35]. This stands in contrast to other studies demonstrating that tongue scraping has an impact on the microbiota at the dorsum of the tongue [33, 36]. Differences in sampling and analytical microbiological methods may explain the differences in results obtained. In the present study, bacterial samples were collected from the dorsum of the tongue using a swab method used for vaginal sampling of bacteria whereas in the study by Roldan et al. [36], the bacteria were sonicated from tongue scrapers. The amounts and diversity of bacteria identified in the present study are consistent with what has been reported elsewhere in regards to the microbiota at the dorsum of the tongue using the same sampling method [42]. The use of a tongue scraper may not be sufficient to

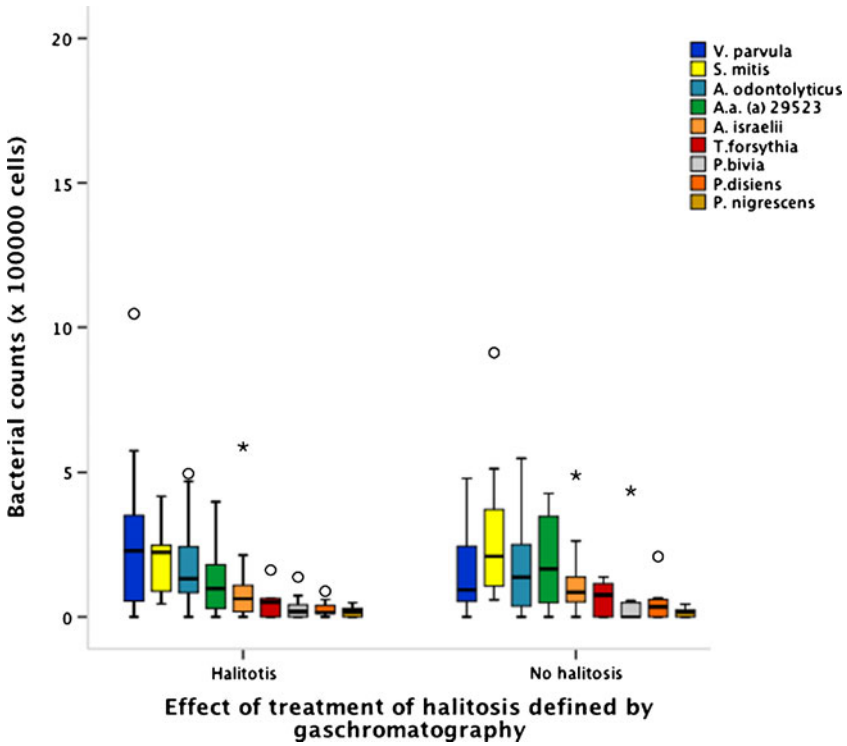

Fig. 3 Boxplot diagram (median values, 25th and 75th percentiles, *empty circle* outlier values, *asterisks* extreme outlier values) presenting bacterial changes in effectively and non-effectively treated subjects in the active rinse and tongue scraping sequence

eliminate bacteria that are located in fissures and crypts of the tongue and from the most dorsal part of the tongue. Tongue scraping may temporarily for some hour remove tongue coating [35, 43]; the bacteria in the tongue biofilm may be able to grow quickly and produce significant amounts of VSCs shortly after scraping the tongue. In a Cochrane review, it was concluded that tongue scraping has limited and short-lived effect on oral halitosis, but also that there are reports of tongue trauma as a consequence of using tongue scrapers [44].

Although subjects were instructed to avoid specific food items, it is possible that food items containing cysteine and methionine may have facilitated the growth of bacteria that produce VSC [45]. In order to reduce VSCs and intra-oral halitosis, it might be necessary to use other means of treatment in subjects with persistent intra-oral halitosis. The present study suggested, however, that active mouth rinse alone was able to control key bacteria associated with intra-oral halitosis.

Several bacterial species that are part of the commensal microbiota in the oral cavity have been associated with both periodontitis and intra-oral halitosis. Analysis of subgingival bacterial samples have shown that abundant producers of VSC include members of the genera *Fusobacterium*, *Campylobacter*, *Prevotella*, *Treponema*, *Eubacterium*, *Selenomonas*, and *Bacteroides* (i.e., *T. forsythia* and *P. gingivalis*) [18, 46, 47]. *Streptococcus salivarius* contributes to intra-oral halitosis by deglycosylating salivary glycoproteins allowing further degradation by Gram-negative organisms [48]. Others have shown that that in addition to *S. salivarius*, *Atopobium parvulum* also present on the dorsum of the tongue contributes to intra-oral halitosis [49]. In older subjects, both *P. melaninogenica* and *F. nucleatum* spp. have been associated with VSCs [50]. The dorsum of the tongue may also harbor other bacterial species that produce VSC. *P. aeruginosa*, one of the bacteria affected by the active rinse alone, is associated with a distinctive smell produced by a combination of volatile compounds including methyl mercaptan [51].

In subjects rinsing with the placebo solution alone, we could not identify any significant effects neither for the reduction of intra-oral halitosis nor for bacterial counts. The adjunct use of a tongue scraper provided limited clinical effects on oral halitosis (85.7 % of the subjects had remaining intra-oral halitosis following this therapy). Consistent with other reports, we found that tongue scraping alone is insufficient for controlling intra-oral halitosis. One reason why the added effects of the tongue scraper are limited might be difficulties in using the device properly. Another explanation may be that bacteria-producing VSCs can be identified also at other locations in the oral cavity or in the oropharygeal area [52–54]

We were able to demonstrate a difference in the levels of bacteria associated with intra-oral halitosis at 14 days in the effectively treated subjects (no halitosis), compared to the subjects that still demonstrated halitosis following the active rinse alone. We were, however, unable to demonstrate a difference in the microflora between the effectively treated (no halitosis) as compared to non-effectively treated individuals at 14 days following the combined treatment of an active rinse and tongue scraping. This difference between the two treatment modalities is an interesting finding while similar improvements in the reduction of oral halitosis were found following these two treatment modalities. One possible explanation may be that tongue scraping reduces the amount of microorganisms on the dorsum of the tongue independent of the outcome on intra-oral halitosis. We noticed a trend of a greater decrease of VSC producing bacteria in subjects who had been effectively treated. The reduction from already low bacterial counts was therefore not sufficient to result in a statistically significant difference between the effectively and non-effectively treated subjects in subjects who had used the active rinse in combination with the tongue scraper.

Zinc salts are US Food and Drug Administration-approved therapeutics with broad applicability as being anti-inflammatory and having antibacterial effects. The explanation to why the active mouth rinse more effectively controlled for VSCs may be the fact that chlorine dioxide- and zinc-containing mouth rinses can neutralize odoriferous sulfur compounds [55, 56]. Oral rinses containing zinc citrate and sodium lauryl sulfate can effectively control the growth of dental plaque [57–62]. Zinc and copper are important in the control and inhibition of co-aggregation of *P. gingivalis*, limiting the settlement of *P. gingivalis* in a biofilm [63]. Furthermore, zinc appears to inhibit the growth of *Fusobacterium* sp. and *Prevotella* spp. [64]. Thus rinsing with a zinc-containing solution can explain the reduction of these species in samples taken at day 14 in the active rinse group. This would also explain the decrease in VSCs.

In conclusion, rinsing with a mouth rinse, containing zinc and chlorhexidine, reduces VSC levels and bacterial counts of several species that may be associated with intra-oral halitosis in subjects without a diagnosis of periodontitis. The adjunct use of the tongue scraper had no adjunct effects on VSC release or in reducing bacterial counts in samples from the dorsum of the tongue in subjects without a diagnosis of periodontitis but with pre-existing intra-oral halitosis.

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