International Journal of Dental Hygiene

Official Journal of the International Federation of Dental Hygienists

ORIGINAL ARTICLE

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Antibacterial action of Chlorhexidine/thymol containing varnishes *in vitro* and *in vivo*

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Abstract: *Objectives:* The antibacterial activity of two different formulations of a chlorhexidine/thymol varnish should be elucidated *in vitro* and *in vivo*. *Methods:* The agar diffusion assay with Cervitec® and CervitecPlus® and three reference strains each of streptococci, lactobacilli, actinomyces and periodontal pathogens was performed. In a split-mouth study, 40 volunteers applied the test (CervitecPlus®, solvent water and ethanol) and control (Cervitec®, solvent ethyl acetate) varnish at buccal recessions of premolar teeth at baseline as well as after two, four and seven days. *Supra-* and subgingival plaques were collected 2 weeks before baseline and at the screening appointments. Supragingival plaque was analysed for mutans streptococci and lactobacilli and subgingival samples for *Aggregatibacter actinomycetemcomitans*, *Fusobacterium nucleatum*, *Porphyromonas gingivalis* and *Porphyromonas intermedia*. Friedman/Wilcoxon tests and *U*-test were used for statistical analysis ($P < 0.05$). *Results:* Most reference strains were susceptible with inhibition zones (mm) as follows: Cervitec®/CervitecPlus® streptococci 27 ± 1.7/21.3 ± 2.5, lactobacilli 26 ± 9.2/23.7 ± 4.9, actinomyces 36.3 ± 6.6/27.3 ± 1.5, periodontal pathogens 18.7 ± 7.6/18 ± 1.7. Both varnishes reduced significantly the counts of mutans streptococci and lactobacilli in the patients. However, no significant differences were found between test and control sides at any time. The total counts of periodontal pathogens were low. A tendency to higher counts of *A. actinomycetemcomitans* at the control side could be shown; the test side did not harbour significantly higher counts. *Conclusion:* Both varnishes may influence the plaque formation and reduce mutans streptococci in supragingival plaque.

Key words: antibacterial activity; chlorhexidine; dental plaque; *in vitro*; split-mouth study

Dates:
Accepted 28 February 2014

To cite this article:
Int J Dent Hygiene **12**, 2014; 168–173.
DOI: 10.1111/idh.12079
Jentsch HFR, Eckert F-R, Eschrich K, Stratul S-I, Kneist S. Antibacterial action of Chlorhexidine/thymol containing varnishes *in vitro* and *in vivo*.

Introduction

Different varnishes with antibacterial ingredients are used to reduce plaque accumulation on tooth surfaces and/or for prevention and therapy of root caries (1, 2). Growth of dental plaque is significantly inhibited by chlorhexidine (3, 4). Gram-positive bacteria, especially mutans streptococci, and gram-negative bacteria are inhibited by chlorhexidine bacteriostatically, and in higher concentrations, also bactericidally (5, 6). Chlorhexidine as a cation can bind to several oral surfaces such as mucosa, gingiva, pellicle and plaque. In this manner, substantivity is

achieved (5). Chlorhexidine varnishes can be considered as more effective than fluoride varnishes against root caries although the evidence is limited and the strength of recommendation grades as 'weak' (7). Chlorhexidine varnishes are often used as a combination of chlorhexidine and thymol. Thymol is a monoterpene with bactericidal and fungicidal properties. Several plants such as thyme, oregano and savoury contain thymol. Thymol is used also in gynaecology, dermatology and veterinary medicine. Thymol acts via disruption of the electron transfer through cell membranes and the active transport, and causing coagulation of cell constituents (8). Furthermore, thymol has anti-inflammatory and antioxidative properties (9, 10).

The formulation as a varnish containing chlorhexidine and thymol reduces side effects of chlorhexidine such as gustatory disorders or desquamation, and allows for prolonged release of its components (11). Cervitec varnishes reduce significantly the occurrence of caries in the occlusal fissures of the teeth (12). The number of mutans streptococci in the dental plaque of orthodontically treated patients and in saliva was significantly reduced by the application of chlorhexidine/thymol varnishes (13, 14). Dental plaque regrowth is significantly reduced by varnish application, but insufficient mechanical plaque control cannot be fully compensated by varnish application (15). The aim of the present study was to get information on the antibacterial activity of two different formulations of the chlorhexidine/thymol varnish on cariogenic germs and periodontal pathogens *in vitro* and *in vivo*.

Materials and methods

Two varnishes (CervitecPlus®, test, solvent water and ethanol) and (Cervitec®, control, solvent ethylacetate) (both Ivoclar Vivadent GmbH, Ellwangen, Germany) were tested *in vitro* and *in vivo*.

Micobiological methods

In vitro procedures

In vitro the agar diffusion assay was performed with reference strains of streptococci (ST, *S. sanguinis* OMZ 9S, *S. sobrinus* OMZ 176 and *S. mutans* NCTC 10449), lactobacilli (LB, *L casei* IMET 10692, *L. coryniformis* DSM 20001 and *L. plantarum* DSMZ 2601), actinomyces (AC, *A. odontolyticus* R22/580 and W59/1094, *A. naeslundii* ATCC 27044) and periodontal pathogens (PP, *Aggregatibacter actinomycetemcomitans* DSZM 8324, *Fusobacterium nucleatum* DSZM 20482 and *Porphyromonas gingivalis* DSZM 20709). Into reservoirs (∅ 10 mm) in Balmelli agar (10 g tryptose, 5 g yeast extract, 5 g K_2HPO_4, 3 g meat extract, 50 g sucrose, 25 g agar, ad 1000 ml Aqua dest.; pH 7.2) mixed with the reference strains, 0.3 ml each of the varnish was placed. Aqua dest. and chlorhexidine (1%) served as controls in the agar diffusion assay; after an anaerobic incubation of 24 h at 37°C the inhibition zones (IZ, mm) were measured.

Subjects and clinical-microbiologic procedure

For the clinical study, 40 male and female volunteers recruited in a private praxis (F.R.E.) participated in a split-mouth study. The approval of the ethical committee of the Medical Faculty of the University of Leipzig (70/2007) was obtained and the volunteers signed an informed consent.

The inclusion criteria were as follows: age between 35 and 55 years, at least one tooth with buccal gingival recession of 1–2 mm and initial root caries (ICDAS code 1), study teeth without any buccal restoration. Exclusion criteria were gravidity and lactation, intake of antibiotics in the last 3 months before the study and allergy in relation to the ingredients of the varnishes. For the allocation to the test and control side, a randomization table was used by an assistant.

At baseline (0), the probing depths and the recessions were determined at the buccal sites (mesiobuccal, buccal and distobuccal) of the test and control teeth (one left and right premolar each) using a PCP-15-probe (HuFriedy GmbH, Leimen, Germany). *Supra-* and subgingival plaque samples were collected, and professional tooth cleaning was performed. After 14 and 16 days (appointment 1 and 2), *supra-* and subgingival plaque samples were taken again. According to the manufacturer's instructions, the test or control varnish was applied to the root surface in the recession area. 18 days after baseline, the application of the varnish was repeated. Three weeks after baseline *supra-* and subgingival plaque samples were taken again. Five weeks after baseline supragingival plaque was collected. The clinical procedure is given in Table 1.

Supragingival biofilm was collected under relative dryness using a dental spatula (Daniel Kürten GmbH, Solingen, Germany). The spatula was immediately pressed on the agar areas of the Caries Risk Test (CRT®*bacteria*, Ivoclar Vivadent GmbH, Ellwangen, Germany) (16) according to the manufacturer's instructions. The CRTs were incubated at 37°C (Cultur M-Mini-Inkubator, Gaimiz, Switzerland) for 2 days.

Supragingival plaque samples were analysed after preliminary calibration. The colony-forming units (CFU) of mutans streptococci (SM) and lactobacilli (LB) were classified as follows: SM/

Table 1. **Flow chart of clinical procedures**

Time	Appointment	Action
Baseline	0	Measurements of probing depth and recession, collection of *supra-* and subgingival biofilm
Baseline + 14 days	1	Collection of *supra-* and subgingival biofilm, application of varnish
Baseline + 16 days	2	Collection of *supra-* and subgingival biofilm, application of varnish
Baseline + 18 days	3	Application of varnish
Baseline + 21 days	4	Collection of *supra-* and subgingival biofilm
Baseline + 35 days	5	Collection of supragingival biofilm

LB 0 and 1 corresponding to $<10^5$ CFU per sample, and SM/LB 3 and 4 corresponding to $>10^5$ CFU per sample.

Subgingival biofilm was sampled by inserting an endodontic paper point (ISO 50; Roeko, Coltene/Whaldent, Langenau, Germany) into the periodontal pocket until resistance was felt for 30 s. All samples were taken under relative dryness and after the removal of all supragingival plaque and debris. After removing the paper strips and points, they were transferred to tubes placed on ice and stored at −20°C as soon as possible until analysis.

The subgingival plaque samples were analysed using competitive quantitative PCR (K. E.) for *P. gingivalis*, *F. nucleatum* and *P. intermedia* and expressed as percentage of the total bacterial counts. The qualitative PCR for *A. actinomycetemcomitans* was classified by an internal standard as negative (0), weakly positive (1), positive (2) and strongly positive (3). PCR protocols, chemicals and equipment used are described in detail at Rupf *et al.* (17, 18).

Data analysis

The statistical analysis of the clinical and laboratory data was made using SPSS (Vers 15.0, Chicago, IL, USA). For intra- and intergroup testing, non-parametric tests (Friedman test, Wilcoxon test, Mann–Whitney *U*-test, respectively) were used. A level of $P < 0.05$ was considered to be significant.

Results

The majority of cariogenic bacteria and periodontal pathogens were susceptible to the varnishes *in vitro*. The mean inhibition zones (mm) were as follows: Cervitec® - streptococci 27 ± 1.7, lactobacilli 26 ± 9.2, actinomyces 36.3 ± 6.6, periodontal pathogens 18.7 ± 7.6; CervitecPlus® - streptococci 21.3 ± 2.5, lactobacilli 23.7 ± 4.9, actinomyces 27.3 ± 1.5, periodontal pathogens 18 ± 1.7 (Fig. 1). The strain-related results are given in Figs 2–5. The inhibition of *S. sobrinus* was higher than for all other tested streptococci (Fig. 2). Considering the lactobacilli strains, the inhibition of *L. casei* was the highest (Fig. 3). The antibacterial activity of Cervitec® against actinomyces was stronger than CervitecPlus® (Fig. 4). The periodontal pathogens showed the smallest inhibition zones. The most sensitive strains were *P. gingivalis* and *F. nucleatum*. The activity of Cervitec® was stronger (exception: *A. actinomycetemcomitans*, Fig. 5).

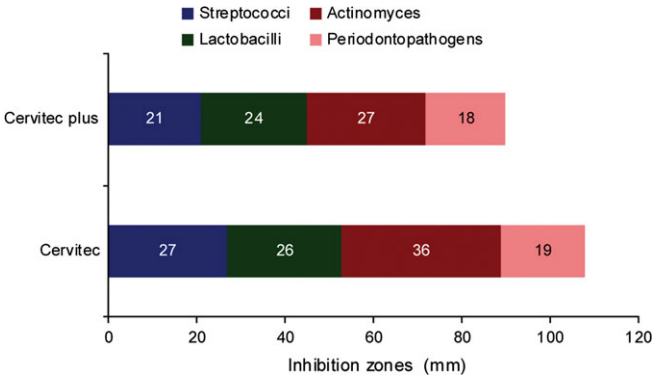

Fig. 1. Inhibition zones (mm) by Cervitec® and CervitecPlus® in the bacterial lawns of streptococci, lactobacilli, actinomyces and periodontal pathogens.

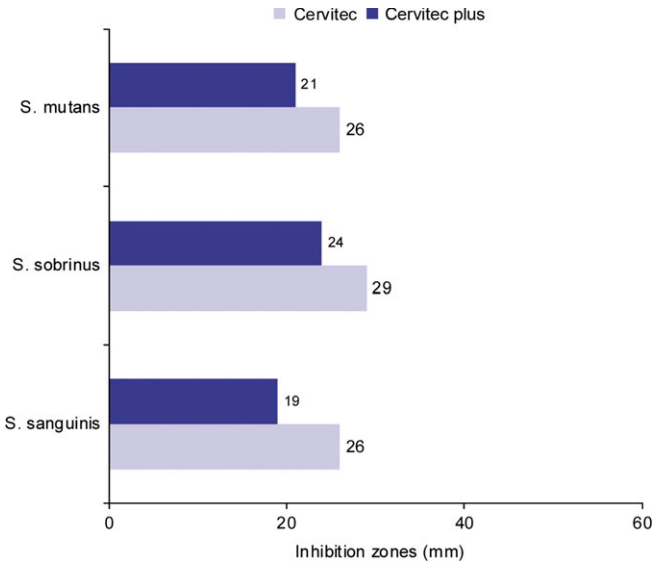

Fig. 2. Inhibition zones (mm) by Cervitec® and CervitecPlus® in the bacterial lawns of *Streptococcus mutans*, *S. sobrinus* and *S. sanguinis*.

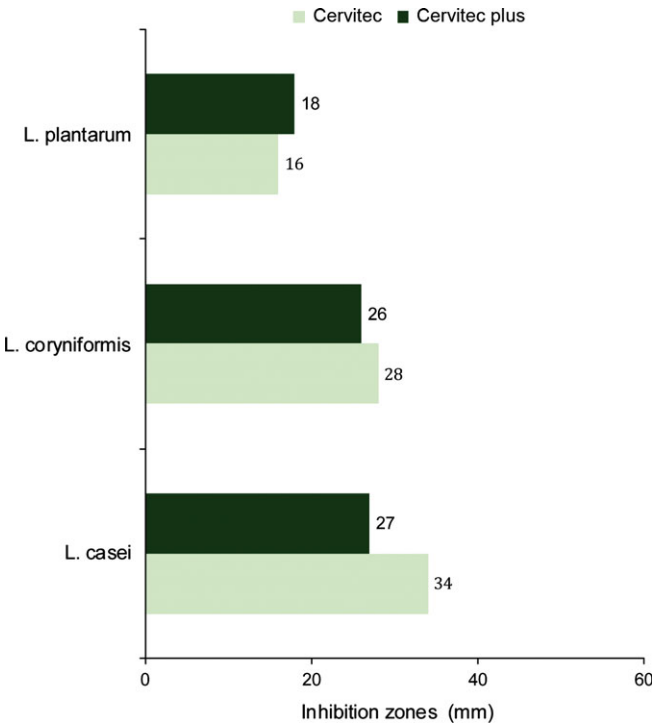

Fig. 3. Inhibition zones (mm) by Cervitec® and CervitecPlus® in the bacterial lawns of *Lactobacillus plantarum*, *L. coryniformis* and *L. casei*.

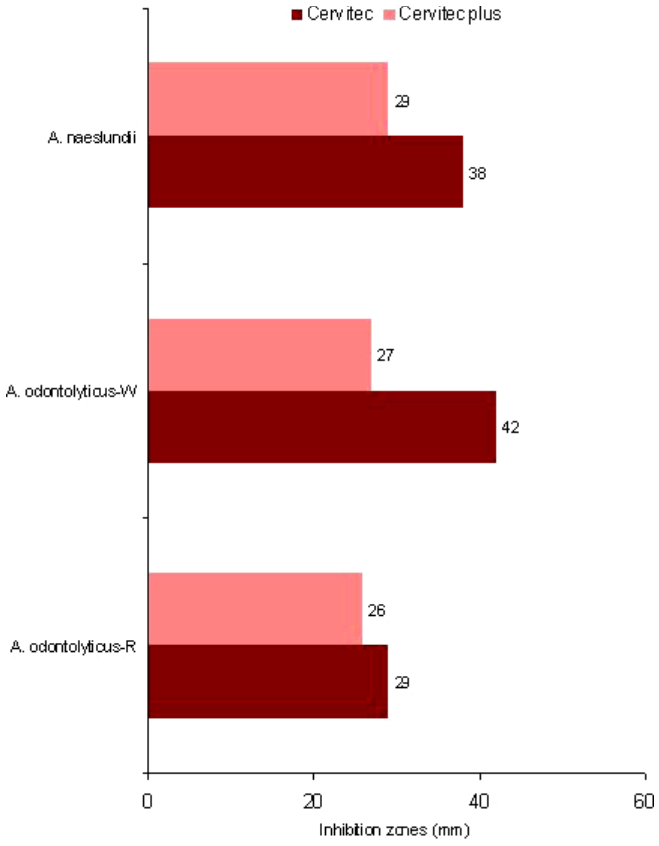

Fig. 4. Inhibition zones (mm) by Cervitec® and CervitecPlus® in the bacterial lawns of *Actinomyces naeslundii* and *A. odontolyticus*.

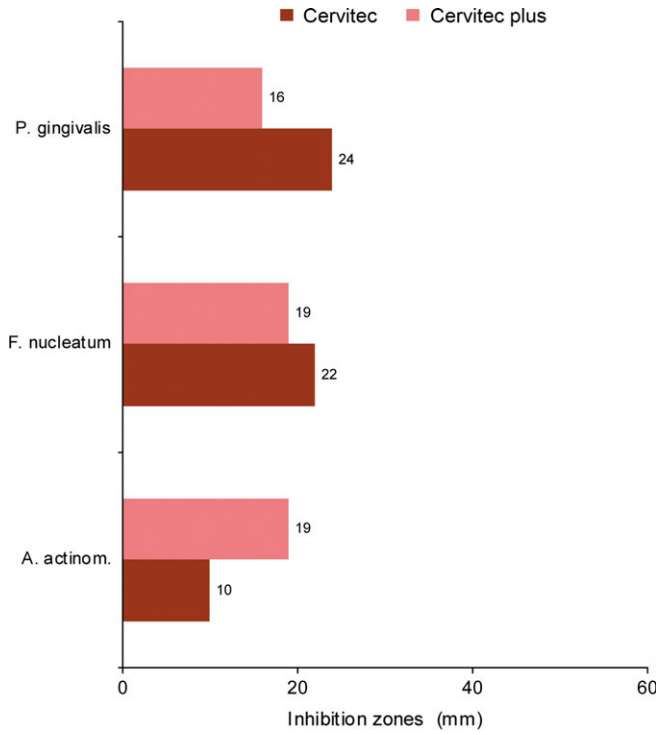

Fig. 5. Inhibition zones (mm) by Cervitec® and CervitecPlus® in the bacterial lawns of *Porphyromonas gingivalis*, *Fusobacterium nucleatum* and *Aggregatibacter actinomycetemcomitans*.

The demographic data of the volunteers and the clinical parameters of the study teeth are given in Table 2. At both sides, significant changes of the CFU of mutans streptococci and lactobacilli occurred during the study. No significant difference to baseline was found at appointment two. At all following appointments, significantly improved results in comparison with baseline were found. No significant differences were found between test and control sides for mutans streptococci and lactobacilli at any time (Table 3).

The results for *P. gingivalis*, *F. nucleatum* and *P. intermedia* were not significant. These results are not given in a Table. The results for *A. actinomycetemcomitans* are presented in Table 4. Inter- and intragroup testing was without significance. The results of the tests side are slightly higher at the end of the study.

Discussion

The aim of the study was to compare the antibacterial activity of two varnishes with chlorhexidine and thymol *in vitro* and *in vivo*. Both varnishes contain each 1% chlorhexidine and thymol referred to the dry substance. The difference between both varnishes was the solvent. At the test side, the solvent was a mixture of water and ethanol; at the control side, the solvent was ethyl acetate. To follow the idea of a split-mouth design, no true control group was included. The binding of the active ingredients in the varnish reduces side effects and results in a prolonged effect for the product of up to 3 months. The combination of chlorhexidine and thymol might potentiate the activity of each single active substance of the varnish (19).

The *in vitro* and *in vivo* results demonstrate clearly that both varnishes have a significant influence on some oral bacteria in the biofilm. The varnish did not significantly influence the growth of bacteria in the subgingival biofilm, especially the mutans streptococci were reduced after the application of the varnish. This is in accordance with other studies using varnishes with different concentrations of chlorhexidine (20, 21) but not with the study by Simoes Moraes *et al.* (1). Furthermore, repeated varnish applications are suggested as tested in our study. The lactobacilli were always in low concentration in our study. The significant reduction in lacto-

Table 2. **Demographic data**

Subjects	Female	Male	Smoker	Non-smoker
40	18	22	18	22
Age/range (years)				
45.5 ± 6.1/ 35–55	43.8 ± 5.2/ 35–53	46.9 ± 6.4/ 35–55		

Parameter	Test side	Control side
Probing depth (mm)		
Mesiobuccal	3.4 ± 0.9	3.3 ± 0.7
Buccal	2.6 ± 0.9	2.6 ± 0.6
Distobuccal	3.5 ± 0.8	3.6 ± 0.6
Recession (mm)	2.1 ± 0.8	2.1 ± 0.7

Table 3. **Categories of CFU (colony-forming unit) of mutans streptococci and lactobacilli of the supragingival plaque at test and control teeth (# Friedman test $P < 0.05$, * Wilcoxon test per appointment to baseline $P < 0.05$)**

Appointment	CFU test				Control				*U*-test, *P*
Mutans streptococci (SM)									
	SM 0	SM 1	SM 2	SM 3	SM 0	SM 1	SM 2	SM 3	
0	14	18	6	2	19	10	7	4	0.921
1	2*	16	16	6	2*	19	15	4	0.427
2	16	19	5	0	15	18	7	0	0.839
4	26*	12	2	0	28*	12	0	0	0.311
5	32*	8	0	0	32*	8	0	0	1.000
Friedman test	#				#				
Lactobacilli (LB)									
	LB 1	LB 2	LB 3	LB 4	LB 1	LB 2	LB 3	LB 4	
0	24	11	3	2	21	13	6	0	0.451
1	6*	28	5	1	6*	23	9	2	0.326
2	22	16	2	0	24	14	2	0	0.738
4	33*	6	1	0	32*	7	1	0	0.914
5	0*	32	7	1	1*	29	10	0	0.895
Friedman test	#				#				

Table 4. **Prevalence of *Aggregatibacter actinomycetemcomitans* in the subgingival plaque at test and control teeth (Friedman test $P < 0.05$, Wilcoxon test per appointment to baseline $P < 0.05$)**

	Category Test				Control				
Appointment	0	1	2	3	0	1	2	3	*U*-test, *P*
0	30	5	4	1	23	4	9	1	0.405
1	23	5	12	0	21	5	13	1	0.783
2	21	5	12	2	27	5	8	1	0.127
4	21	8	10	1	24	6	9	0	0.593
Friedman test	n.s.				n.s.				

n.s., non-significant.

bacilli during and at the end of the study is in accordance with results by Baygin *et al.* (22). In comparison with mutans streptococci, lactobacilli require higher concentrations of chlorhexidine, and the inhibitory effect is dose-dependent (23, 24). The treatment with varnishes containing, for example, chlorhexidine is challenging regarding the recolonization (25, 26). Jenatschke *et al.* showed that even after repeated application of a chlorhexidine varnish, a reduction in mutans streptococci occurred but a recolonization was seen at the end of the study (27).

The present *in vivo* study could not demonstrate an effect on subgingival bacterial species. This fact is not surprising because a distance effect cannot be expected. On the other hand, this could mean that just the plaque in the varnished area, but not in the adjacent regions, is affected by the varnish. No patients with periodontitis were included in this study, and the probing depth beside the application area of the varnish was low. Just a nonsignificant slight increase in the bacterial counts of *A. actinomycetemcomitans* was seen.

It is not proven that fluoride or chlorhexidine varnishes result in higher reductions in mutans streptococci, actinomyces or lactobacilli on root surfaces than professional tooth cleaning (28, 29).

One may conclude that both varnishes are able to reduce the bacterial counts of mutans streptococci and lactobacilli in supragingival plaque. The use of the test varnish which does not contain the solvent of the control varnish does not result in a reduced antibacterial activity during repeated application in a 5-week period.

Clinical relevance

Scientific rationale for the study

To demonstrate the antibacterial effectiveness of a new formula of a chlorhexidine and thymol containing varnish *in vitro* and *in vivo*.

Principal findings

Regarding the clinical outcome, there are no significant differences between the varnishes with the test solvents water and ethanol in comparison with the control solvent ethyl acetate.

Practical implications

The varnish with the solvents water and ethanol can be recommended to modify plaque composition.

Acknowledgements

The authors are grateful to Katrin von Brandenstein and Regina Mäuer (Biological Laboratory, Clinic for Prosthetic Dentistry and Dental Materials, Centre of Dentistry, University Hospital of Jena, Jena, Germany) for technical assistance.

Conflict of interest and sources for funding

The authors declare that they have no conflict of interest. The study was supported by Ivoclar Vivadent GmbH, Ellwangen,

Germany, who provided the varnishes and financially supported laboratory analyses.

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