

# Microbiological and Clinical Effects of a Proanthocyanidin-enriched Extract from *Rumex acetosa* in Periodontally Healthy Carriers of *Porphyromonas gingivalis*: a Randomized Controlled Pilot Study

## Authors

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## Key words

*Rumex acetosa*, Polygonaceae, proanthocyanidines, anti-adhesive, *Porphyromonas gingivalis*

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## ABSTRACT

*Rumex acetosa* significantly inhibits the adhesion of *Porphyromonas gingivalis* (*P.g.*) to eukaryotic host cells *in vitro*. The objective of this randomized placebo-controlled pilot-trial was to analyze effects of a mouth rinse containing 0.8% (w/w) of a quantified proanthocyanidin-enriched extract from *Rumex acetosa* (RA1) on microbiological, clinical, and cytological parameters in systemically healthy individuals without history of periodontitis, harboring *P.g.* intraorally. 35 subjects received a supragingival debridement (SD) followed by mouth rinsing (3 times daily) with either RA1 mouth rinse solution (test) or placebo (control) for 7 days as adjunct to routine oral hygiene. Supragingival biofilm samples were taken at screening visit, baseline (BL), 2, 4, 7 and 14 days after SD. *P.g.* and 11 other oral microorganisms were detected and quantified by rtPCR. Changes in the oral microbiota composition of one test and one control subject were assessed via high throughput 16S rRNS gene amplicon sequencing. Approximal Plaque Index (API) and the modified Sulcular Bleeding Index (SBI) were assessed at BL, 7- and 14-days following SD. Brush biopsies were taken at BL and 14 d following SD. Intergroup comparisons revealed no significant microbiological, cytological, and clinical differences at any timepoint. However, a significant reduction in SBI at day 14 ( $p = 0.003$ ) and API at day 7 ( $p = 0.02$ ) and day 14 ( $p = 0.009$ ) was found in the test group by intragroup comparison. No severe adverse events were observed. The results indicate that RA1 mouth rinse is safe but does not seem to inhibit colonization of *P.g.* or improve periodontal health following SD.

## Introduction

*Porphyromonas gingivalis* (*P.g.*) is one of the major pathogens associated with the onset and progression of periodontitis [1]. It is viewed as a “Keystone Pathogen” in altering the commensal oral microbiota towards a dysbiosis that contributes to the detrimental clinical effects of periodontitis [2, 3]. *P.g.* is not only detected in patients with periodontal disease, but can frequently be detected

in periodontally healthy individuals, which may present a microbiological risk factor for periodontitis [1, 3, 4]. Repeated professional supragingival plaque removal markedly reduces bacterial counts but fails to eradicate *P.g.* from supra- and subgingival habitats [5]. The adhesion of *P.g.* to oral surfaces, primarily mediated by fimbriae, hemagglutinins and gingipains [6], is pivotal for its colonization and consecutive transformation of the indigenous healthy oral microbiome, specifically modifying the prevalence of

## ABBREVIATIONS

API	aproximal Plaque Index
<i>P.g.</i>	<i>Porphyromonas gingivalis</i>
RA1	quantified proanthocyanidin-enriched extract from <i>Rumex acetosa</i>
SBI	sulcular Bleeding Index
SD	supragingival debridement

other periodontal pathogens, i.e. *Aggregatibacter actinomycetem-comitans* (*A.a.*), *Treponema denticola* (*T.d.*), *Tannerella forsythia* (*T.f.*), *Prevotella nigrescens* (*P.n.*), *Prevotella intermedia* (*P.i.*), *Eikenella corrodens* (*E.c.*), and caries associated bacteria like *Streptococcus mutans* (*S.mutans*). Inhibiting the adhesion of *P.g.* in the first instance presents a cytoprotective strategy and is a novel approach in treating infections [7] and potentially preventing periodontitis or caries.

Plant-derived polyphenols show potential in the treatment and prevention of periodontitis [8]. Especially oligomeric proanthocyanidins (OPC), characterized by oligomerized flavan-3-ol building blocks, exhibit strong anti-inflammatory and anti-adhesive effects [9, 10]. OPC containing extracts from green tea (*Camellia sinensis*) [11], *Myrothamnus flabellifolia* [12], cranberry fruits (*Vaccinium macrocarpon*) [13–15], pomegranate fruits (*Punica granatum* L.) [16], *Rhododendron ferrugineum* [17] and *Limonium brasiliense* [18] have shown to effectively inhibit the adhesion of *P.g.* to human epithelial host cells. Moreover, an OPC-enriched and quantified extract from common sorrel (*Rumex acetosa* L., RA) has been demonstrated *in vitro* to significantly inhibit the adhesion of *P.g.* [19] to human buccal cells in a dose-dependent manner of up to 90% [20]. Epicatechin-3-*O*-gallate-(4 $\beta$ -8)-epicatechin-3-*O*-gallate (procyanidin-B2-di-gallate) has been identified as main antiadhesive compound, which strongly interacts with the bacterial virulence factor Arg-gingipain of *P.g.*, while the corresponding Lys-gingipain was hardly influenced. *In silico* docking studies revealed that the galloylation of this compound is responsible for the fixation of the ligand to the Arg-gingipain [20]. Systematic preclinical investigation of RA indicated that the extract not only interferes with the attachment of *P.g.* to eukaryotic host cells, but also inhibits the adhesion of *Herpes simplex* 1 [21] and influenza A virus to host cells *in vitro* [22]. As plant extracts are usually complex mixtures of different natural products the phytochemical composition of RA has been investigated in detail, indicating monomeric flavan-3-ols (epicatechin-3-*O*-gallate), oligomeric proanthocyanidins (epicatechin-(4 $\beta$ →8)-epicatechin (syn. procyanidin B2), epicatechin-3-*O*-gallate-(4 $\beta$ →8)-epicatechin-3-*O*-gallate (syn. procyanidin B2-di-gallate), epicatechin-( $\beta$ →8,2 $\beta$ →*O*→7)-epicatechin-(4 $\beta$ →8)-epicatechin (syn. cinnamtannin B1), and flavonoids (quercetin-3-*O*-glucuronide) as the main constituents [23]. The lead compound, interacting with the functionally important regions of the main virulence factors of *P.g.*: Arg-gingipain and hemagglutinin, has been identified as epicatechin-3-*O*-gallate-(4 $\beta$ ,8)-epicatechin-3'-*O*-gallate [20].

The traditional use of common sorrel (*R. acetosa*) for the treatment of inflammatory diseases in the mouth has been described

over centuries. However, no scientific data for rationalizing this ethnopharmacological use have been recorded. Not surprisingly, controlled clinical trials studying the anti-inflammatory and anti-adhesive effects are still missing.

Therefore, a mouth rinse, containing 0.8% (w/w) of a quantified proanthocyanidin-enriched extract of RA was developed (RA1) and evaluated in a randomized controlled pilot-trial for its microbiological, clinical and cytological effects in systemically healthy individuals with a Community Periodontal Index of Treatment Needs CPITN  $\leq 2$  [24] and harboring *P.g.* intraorally.

## Results

The microbiological, clinical and cytopathological effects of either RA1 or the placebo mouth rinse in combination with supragingival debridement, were evaluated in a randomized controlled trial with a follow-up period of 14 days.

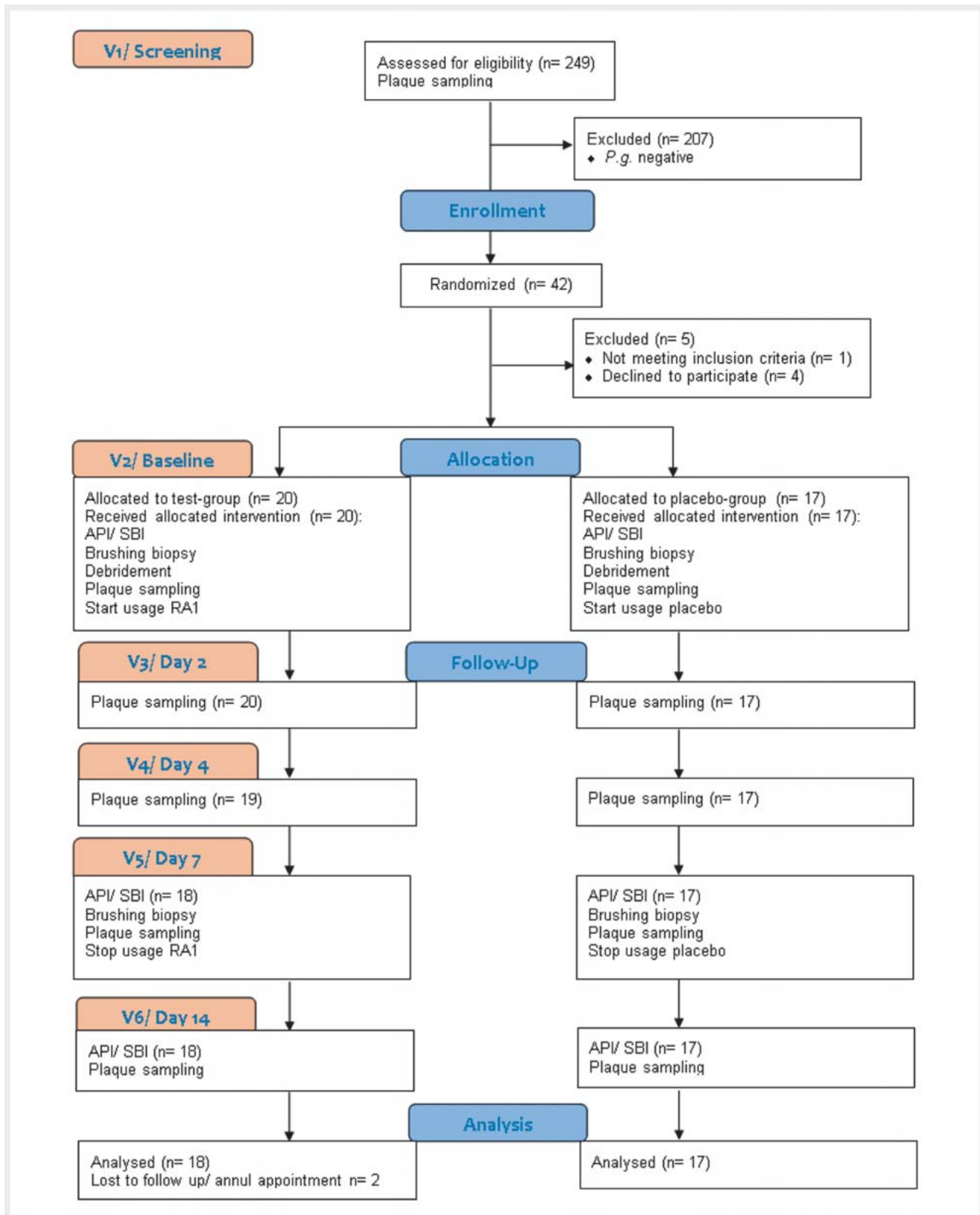
42 out of 249 subjects fulfilling the clinical inclusion criteria were found to harbor *P.g.* intraorally and were enrolled into the study. Prior to baseline visit, 4 subjects withdrew their participation due to scheduling difficulties and 1 subject had to be excluded due to systemic antibiotic therapy. Furthermore, 2 subjects dropped out during the study as they cancelled or did not attend the scheduled appointment. A detailed overview of the participant study flow is presented in ► Fig. 1 and the final group distribution and demographic characteristics in ► Table 1. In terms of age, gender, smoking status, number of teeth and clinical parameters (API and SBI), the study subjects were equally distributed across the two groups.

No significant differences were detected between the test and control group for any of the tested microorganisms at any time point.

► Fig. 2 shows *P.g.* as a relative fraction of the total biofilm count according to the different reference dates. In the control group, a significant reduction of *P.g.* from baseline to day 2 ( $p < 0.01$ ), day 4 ( $p < 0.01$ ) and day 7 ( $p < 0.01$ ) was found, whereas there was no significant change in the test group. The boxplot diagram shows an initial reduction for both groups until day 4, followed by a trend towards an increase and a higher distribution of the individual values within the test group. No significant changes in other putative periodontal pathogens (► Fig. 3a) or commensal bacteria (► Fig. 3b) were found in neither group 7 nor 14 days after debridement.

High throughput 16S rRNA gene sequence analysis was performed on one control (SA120) and one test subject (SA088), at each time-point (V1–V6), to obtain a representative 'snap-shot' of the bacterial microbiome in each subject group (results are presented in the Supporting Information).

No significant differences in the assessed clinical parameters were found between test and control group at any time point throughout the observation period. However, in the test group the API was significantly reduced from baseline to day 7 ( $p < 0.05$ ) and day 14 ( $p < 0.01$ ) (► Fig. 4) while no significant changes over time in the API were observed in the control group. The modified SBI was significantly reduced compared to baseline in the test group at day 7 ( $p < 0.001$ ) and 14 ( $p < 0.01$ ) and in the control group at day 7 ( $p < 0.01$ ), see ► Fig. 4. Further, the boxplot



► **Fig. 1** Flow chart of the study design and participants according to CONSORT 2010.

► **Table 1** Group characteristics. Demographic and clinical characteristics of the included participants at baseline V2.

	Test	Placebo	p-value
Number of participants	18	17	X <sup>2</sup> = 0.866
Age (mean ± SD)	44.83 ± 15.33	41.41 ± 13.31	Mann-Whitney-U-test p = 0.546
Gender F (female), M (male)	F 12, M 6	F 13, M 4	Fisher test p = 0.71
Current smoker	2	2	Fisher test p = 1.000
Number of teeth (mean ± SD)	27.44 ± 2.04	27.88 ± 2.42	Mann-Whitney-U-test p = 0.782
API V2 (mean % ± SD)	46.56 ± 24.32	49.24 ± 22.24	Mann-Whitney-U-test p = 0.708
SBI V2 (mean % ± SD)	28.28 ± 19.37	35.35 ± 23.66	Mann-Whitney-U-test p = 0.351

SD: standard deviation

diagram illustrates a narrow scatter of the individual values for both groups at day 7 and day 14.

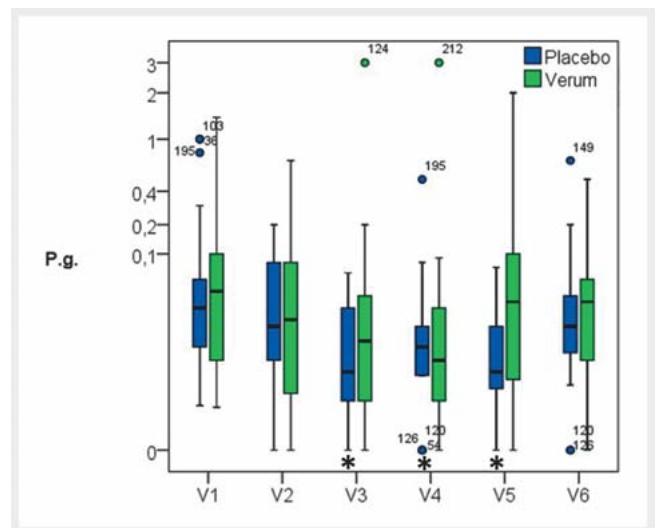
Investigation of cytopathology indicated that neither the verum nor the placebo mouth rinse caused dysplastic changes of oral mucosal cells.

No severe adverse events were recorded during the study period. In the test group, minor complaints including a transient brown discoloration of the tongue (n = 8), a dry mouth feeling after rinsing (n = 4), an unpleasant alcoholic taste of the mouth rinse (n = 4) and a white cloudy consistence of the saliva after rinsing (n = 4) were reported. None of the participants considered breaching the rinsing protocol and the observations vanished completely at day 14.

## Discussion

This study is the first to examine the intraoral effects of a proanthocyanidin-enriched and standardized extract from *R. acetosa* L. on a microbiological, cytopathological and clinical level. RA1 was found to be safe without any observed impact on the composition of the intraoral microbiota. However, RA1 did not significantly reduce the intraoral prevalence of *P.g.*.

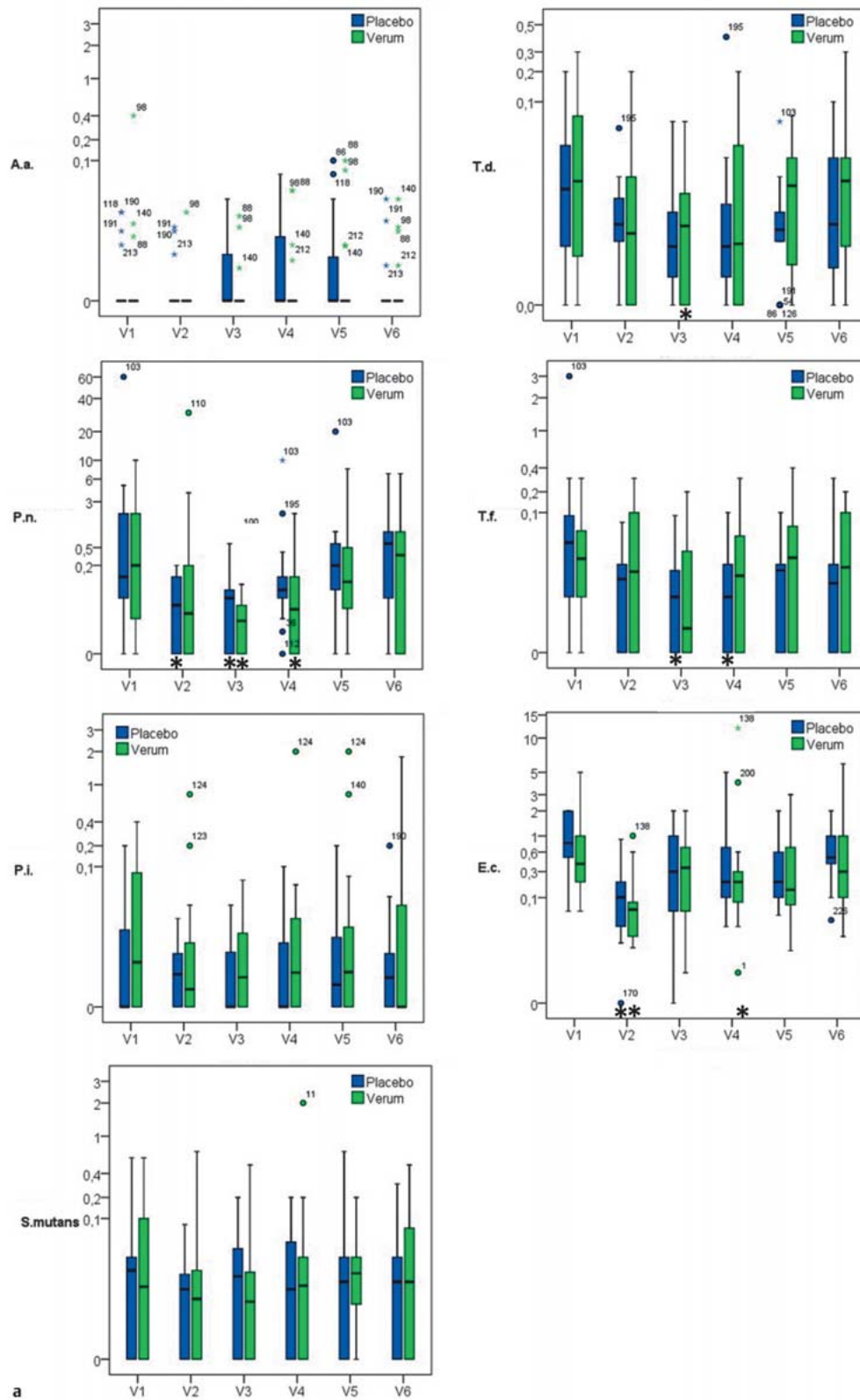
The early microbial succession in redeveloping dental biofilms in periodontally healthy and diseased subjects after professional debridement and followed by 7 days of no oral hygiene has been assessed before [25]. In the study of Teles et al. (2012) [25], the values for *P.g.* within the supragingival biofilm samples showed no significant difference directly after the debridement and 1-day post. Whereas 2, 4 and 7 days later the values were significantly reduced. The same pattern can be seen in the placebo group of this study with a significant reduction of *P.g.* at day 2, day 4 and day 7. In contrast, *P.g.* values for the test group did not show any significant reduction. Looking at day 7 in the boxplot diagram (see ► **Fig. 2**) it even shows an increased variability of the individual values. Noting that the *P.g.* levels vary differently in those two groups, across the time-points, it can be concluded that RA1 has an effect on *P.g.* *in vivo* during this period. Theoretically, *P.g.* might initiate regulatory or compensatory mechanisms, e.g., changing its proliferation rate or increasing the expression of adhesion proteins, to counteract the adhesion inhibition by RA1.



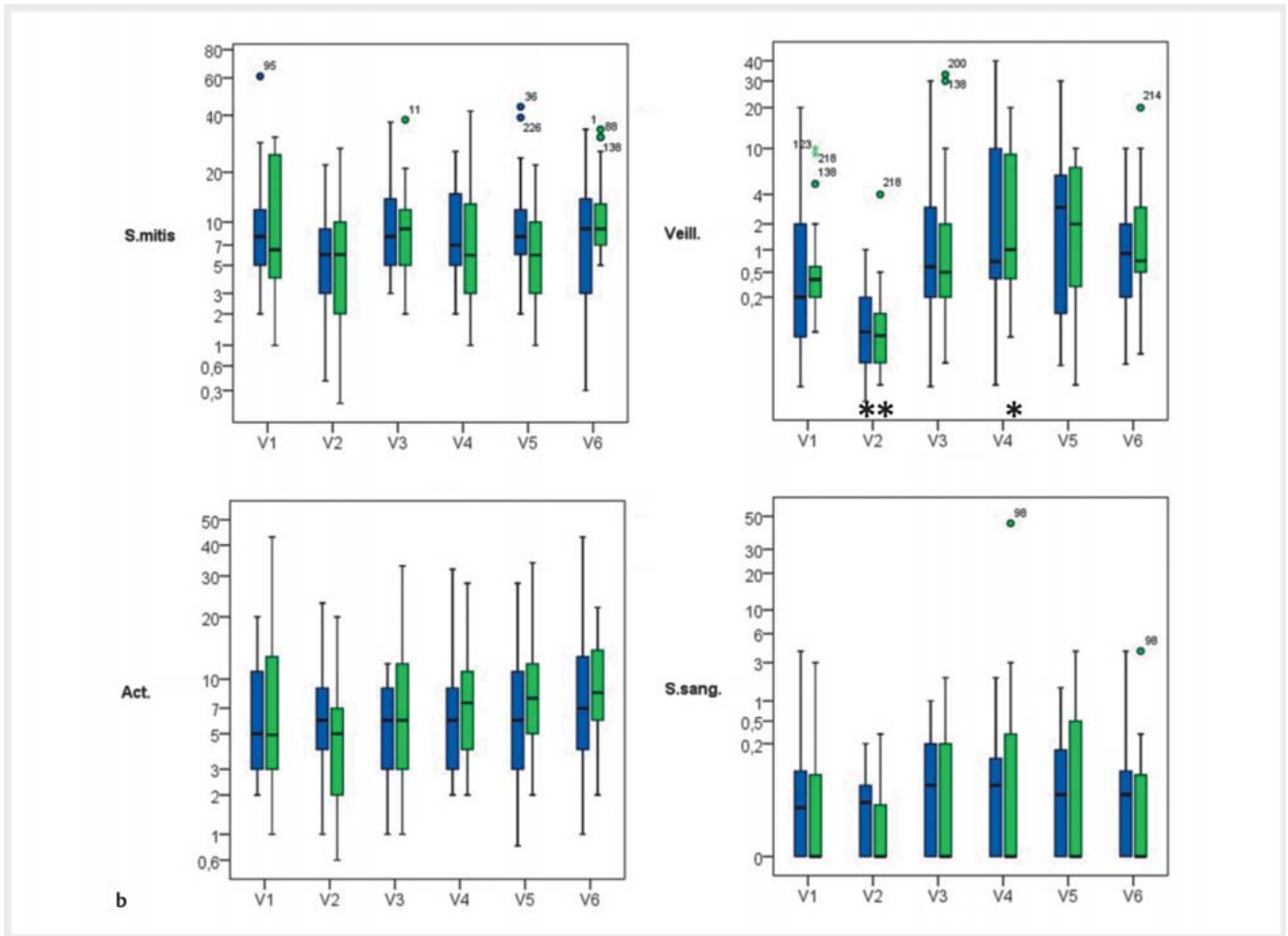
► **Fig. 2** Prevalence of *P.g.* (relative fraction of the total plaque count) according to the time of measurement (V1 = screening, V2 = baseline, V3 = day 2, V4 = day 4, V5 = day 7, V6 = day 14) as a boxplot showing 0th, 25th, median, 75th and 100th percentile, non-linear display of the Y axis to the exponent 0.2. Significant changes between the V2–V6 time-points compared to the V1 time-point are marked with an asterisk symbol.

*In vitro* studies on the influence of RA on the expression of *rgpA* for Arg-gingipain, *kgp* for Lys-gingipain and *fimA* for fimbrillin demonstrated constant gene expression rates in *P.g.* liquid cultures [20]. It is known that the gene expression of *P.g.* differs when grown in a biofilm or planktonic culture [26]. Hence, additional *in vitro* studies should investigate the effects of RA1 on gene expression levels when *P.g.* is biofilm associated.

The prevalence of the other pathogenic bacteria tested, *A. a.*, *T. d.*, *P. n.* and *S. mutans*, parallels those described by Teles et al. (2012) [25]. Interestingly, the results seem to differ for *T. f.*. Whereas the placebo group follows the same significant reductive trend as seen in the study from Teles et al. (2012) [25], the values for the test group seem to stay at a constant level. In supra- and



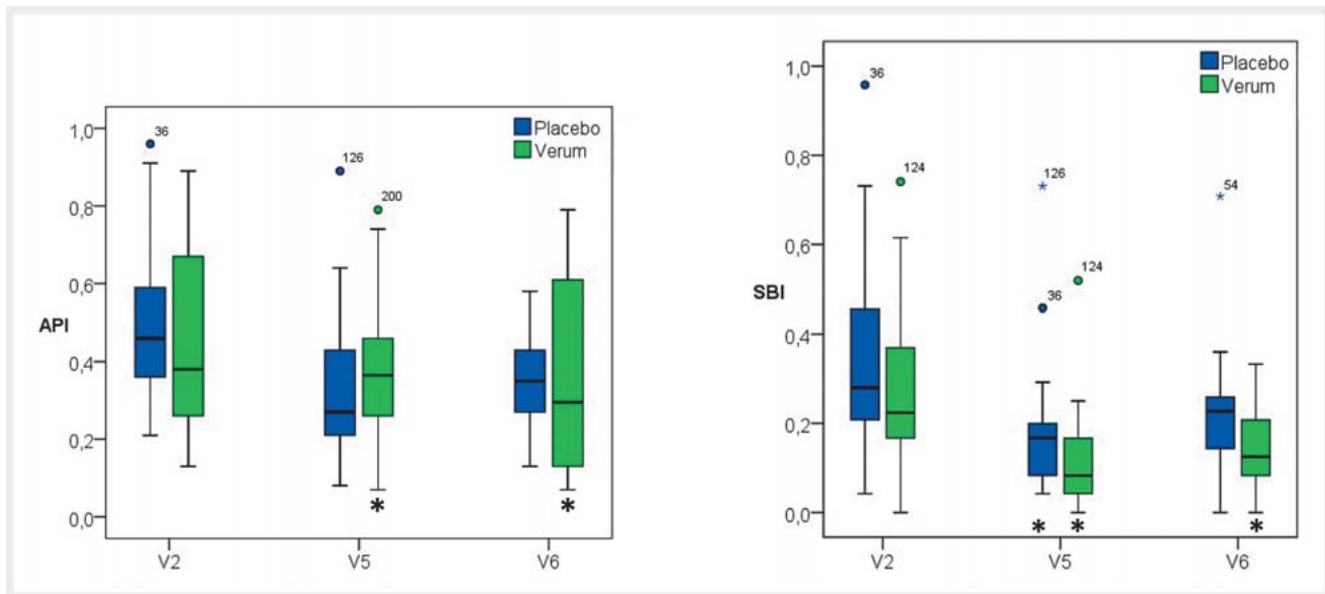
► **Fig. 3 a** Prevalence of 8 oral pathogenic microorganism (relative fraction of the total plaque count) according to the time of measurement (V1 = screening, V2 = baseline, V3 = day 2, V4 = day 4, V5 = day 7, V6 = day 14) as a boxplot showing 0th, 25th, median, 75th and 100th percentile, non-linear display of the Y axis to the exponent 0.2. Significant changes between the V2–V6 time-points compared to the V1 time-point are marked with an asterisk symbol.



► **Fig. 3 b** Prevalence of 4 commensal bacteria (relative fraction of the total plaque count) according to the time of measurement (V1 = screening, V2 = baseline, V3 = day 2, V4 = day 4, V5 = day 7, V6 = day 14) as a boxplot showing 0th, 25th, median, 75th and 100th percentile, non-linear display of the Y axis to the exponent 0.2. Significant changes between the V2 to V6 time-points compared to the V1 time-point are marked with an asterisk symbol.

subgingival biofilms *P.g.* and *T.f.* cells may associate with one another [27,28] and both have a synergistic effect in causing periodontal abscesses [29]. Under limited nutrient supply *T.f.* stimulates the growth of *P.g.*, and gingipains play an important role in the uptake and digestion of growth-promoting factors [29]. *T.f.* likewise has the ability to adhere and invade into epithelial cells and the presence of *P.g.* enhances this process [30]. For the commensal bacteria *S. sang.*, *Veill.*, *S. mitis* and *Act.* the values of the test and placebo groups are akin to those reported by Teles et al. (2012) and no intergroup differences could be detected. *In vitro* tests have shown that proanthocyanidins selectively target gram-negative periodontal pathogenic strains while preserving the viability of the beneficial commensal *Streptococcus salivarius* [31]. RA1 and the respective placebo do not cause a significant overgrowth of pathogenic bacteria or a significant reduction of commensal bacteria. The control and test mouth rinses have no negative effect on the qualitative composition of the supragingival biofilm and the exemplary microbiome analysis of one test and one control subject validated this.

PCR does not differentiate between viable and dead bacteria. If a mouth rinse has a fast therapeutic effect on reducing the amount of living pathogens, using a molecular genetic analysis via real time quantitative PCR would have been a desired method [32]. RA1 was not designed with an antibacterial concentration. Therefore, determination of the viability of *P.g.* was not requisite. For this study a baseline professional debridement was performed to disrupt the existing biofilm and to enable RA1 to directly interact with *P.g.* and other bacteria. In general, supragingival debridement reduces the basic bacteria count to a uniform level and after two days the total bacteria count is expected to correlate with the initial values again [25,33]. Hence, any anti-adhesive and biofilm inhibitory effect of RA1 should be reflected in changes of clinical and microbiological parameters after 2 days, as it has been demonstrated with pomegranate and green tea-containing mouth rinses [16,34]. The professional debridement induced the overall reduction of the API values in both groups at the day 7 time-point. At day 14, the API for the placebo group reverted to the baseline values. In contrast, the test group's proximal biofilm levels contin-



▶ **Fig. 4** API and SBI in percentage (0.4 = 40%) according to the time of measurement (V1 = screening, V2 = baseline, V3 = day 2, V4 = day 4, V5 = day 7, V6 = day 14) as a boxplot showing 0th, 25th, median, 75th and 100th percentile. Significant changes between the V2 to V6 time-points compared to the V1 time-point are marked with an asterisk symbol.

ued to stay significantly decreased even 7 days after cessation of RA1. It seems that RA1 has a prolonged inhibitory effect on biofilm formation. In contrast to this finding, long term studies with a cranberry mouth rinse over 6 weeks failed to prove a biofilm inhibitory effect but noticed a reduction in the total bacteria count [35]. Further long-term studies are needed to determine if bacteria build up a tolerance against the mouth rinse, and for how long RA1 remains effective after cessation.

Using a CPITN  $\leq 2$  as inclusion criteria implies that participants with gingivitis have been included into the study. The SBI values at baseline signal a modest level of gingival inflammation in both groups and those numbers are coherent with the amount of biofilm detected in the API at the same time-point. In systemically healthy patients, debridement improves clinical signs and symptoms of gingival inflammation within 14 days. Therefore, the main cause of the significant reduction of the sulcular bleeding at day 7 in both groups seems to be the removal of calculus and biofilm at the baseline visit. *In vitro*, RA has been shown to significantly reduce IL-6 release and therefore act cytoprotective [19]. As the SBI in the test group on day 14 continued to stay on a significantly reduced level, this could either be based on the lower biofilm levels or on the reduced level of the proinflammatory cytokine IL-6. Future studies involving a larger number of participants will be required to determine whether RA1 reduces sulcular bleeding levels or the plaque formation by a significant amount. In addition, an experimental gingivitis study model (or similar) may be highly informative for further elucidating the potential influence of RA1 on proinflammatory cytokines [36,37].

To validate the effectiveness of a mouth rinse and the method of testing, a test substance should be assessed against a positive and a negative control rinsing agent. 0.2% chlorhexidin digluconate is the 'gold-standard' due to its unspecific bacteriostatic

and subsequent plaque inhibiting effect. Nevertheless, the focus of this study was to determine the anti-adhesive properties of RA1 versus a placebo and not the antimicrobial effect. Hence, it was decided not to test against a positive control group using chlorhexidin digluconate.

It has already been demonstrated that proanthocyanidins are non-toxic to gastric mucosal cells [38]. Nevertheless, mouth rinse formulas can have a mutagenic and cytotoxic effect on mucosa cells [39]. Therefore, assessing the safety of RA1 on a cytologic level was indispensable. Brush biopsies with a negative cytology and a lack of DNA aneuploidy can exclude high-grade oral epithelial cell dysplasia or squamous cell carcinoma avoiding invasive diagnostic biopsies [40]. In this study none of the participants of either group showed dysplastic changes of the buccal mucosa cells. It follows, RA1 is non-cytotoxic and safe to use over a 7-day period.

Proanthocyanidins are commonly linked to a bitter taste, an astringent sensation and the formation of complexes (precipitates) of salivary proteins + procyanidin [41]. The participant's compliance to the rinsing protocol could have been affected by this. A few members of the test group reported only minor complaints, which did not affect their quality of life during the study period or prevented from adhering to the rinsing protocol. All experienced sensations can be related to the proanthocyanidins' characteristics. Therefore, the test mouth rinses can be considered non-cytotoxic over a clinical usage period of 7 days and in addition to that is well tolerated by the study participants.

Although the RA1 mouthwash did not show any therapeutic effects by intergroup comparisons, the intragroup comparison indicates anti-inflammatory effects and interference with biofilm formation. This and the lack of any cytotoxic effects may support the notion of RA1 as a safe cosmetic adjunct following professional

debridement. However, long-term intervention studies are needed to fully appraise the anti-inflammatory and anti-plaque effects of *R. acetosa*.

## Material and Methods

### Study subjects

This study has been carried out in accordance with the ethical principles of the World Medical Association Declaration of Helsinki (version 2008) and was independently reviewed and approved by the institutional review board of the Medical Faculty of the Heinrich-Heine-University, Düsseldorf, Germany (approval number #3786, approval date 16/03/2012). The clinical trial was registered at [www.clinicaltrials.gov](http://www.clinicaltrials.gov) under the number NCT02039648. All participating individuals signed an informed consent form. Inclusion criteria were age  $\geq 18$  years, systemically healthy, a CPITN  $\leq 2$  and tested positive for *P.g.* (see below for experimental details). Exclusion criteria were age  $< 18$  years, pregnancy or nursing, allergies to several mouthwash components, soft tissue lesions (e.g. leucoplakia, lichen planus), any systemic conditions that require an antibiotic prophylaxis for routine dental procedures (e.g. endocarditis), antibiotic therapy in the previous 6 months, any systemic disease (e.g. diabetes or immunological disorders) or long-term medication, that might have potential influence on the immune response, history of periodontal disease and a CPITN  $\geq 3$ .

### Study design

The microbiological, clinical and cytopathological effects of either RA1 or the placebo mouth rinse in combination with supragingival debridement, were evaluated in a two-arm parallel randomized controlled clinical trial with a follow-up period of 14 days. The participants, the examiner and the outcome assessors were blinded to the group assignment.

The extract RA1 was prepared from the herbal material of *R. acetosa* L., as previously described in detail [20]. In principle dried *R. acetosa* herbal material (A. Galke, batch 14593) was exhaustively extracted with acetone/water (7:3 v/v), followed by evaporation of the organic solvent of the extract *in vacuo*, removal of precipitated chlorophyll by filtration, removal of lipophilic compounds by extraction with heptan and lyophilisation of the aqueous phase to yield 9.3% (w/w) of the extract RA1. For analytical quality control a ICH-2 validated UHPLC method [20] was used for identification and quantitation of the marker compounds epicatechin-3-O-gallate (17.9 mg/g extract RA1), epicatechin-3-O-gallate-(4 $\beta$ -8)-epicatechin-3-O-gallate (syn. procyanidin-B2) (3.8 mg/g), epicatechin-3-O-gallate-(4 $\beta$ -8)-epicatechin-3-O-gallate (syn. procyanidin-B2-di-gallate) (11.9 mg/g), epicatechin-(4 $\beta$ -8,2 $\beta$ -O-7)-epicatechin-(4 $\beta$ -8)-epicatechin (syn. cinnamtannin B1) (6.0 mg/g) and quercetin-3-O-glucuronide (24.0 mg/g) [20].

Mouth rinse solutions, containing 0.8% (w/w) of RA1 were manufactured in a facility, which has been approved for manufacture of pharmaceuticals. The detailed composition of the verum and placebo preparations are displayed in ► **Table 2**. All ingredients complied with the specifications of European Pharmacop

peia. Food coloring and aroma were added to the placebo mouth rinse to mimic the taste, smell, and color of the test solution.

The rinses were stored in identical containers and labeled with numbers following a randomly assigned allocation ratio of 50:50 by a staff-member of the Institute of Pharmaceutical Biology and Phytochemistry, University of Münster, Germany. The mouth rinses were handed to the subjects in ascending sequence of their label number following the baseline date.

The treatment allocation was unknown to the examiner, the study participants, microbiologist and cytopathologist. A code break for the randomization was stored in individually sealed envelopes on site in case of adverse events. The randomization scheme was decoded for statistical analyses.

### Intervention

At baseline, the participants received a supragingival debridement (SONICflex 2003 L with Paro instrument tip No. 60, KaVo) and polishing (AIR-FLOW MASTER with AIR-FLOW Powder Perio and Classic, EMS). Standardized instructions were given, in verbal and written form, to rinse 3 times per day with 10 ml of the assigned mouth rinse for 7 days in addition to their oral hygiene procedures. No specific oral hygiene instructions were provided. Supragingival biofilm samples were taken at pre-determined visits (V1 = screening, V2 = baseline after debridement, V3 = 2 days, V4 = 4 days, V5 = 7 days and V6 = 14 days after baseline) and *P.g.* was identified and quantified by real-time polymerase chain reaction (qrt-PCR) using previously described primers (Supplementary Data **Table 1S**). In addition, the amounts of eight species of potentially-pathogenic oral bacteria: *Aggregatibacter actinomycetemcomitans* (*A.a.*), *Treponema denticola* (*T.d.*), *Tannerella forsythia* (*T.f.*), *Prevotella nigrescens* (*P.n.*), *Prevotella intermedia* (*P.i.*), *Eikenella corrodens* (*E.c.*) and *Streptococcus mutans* (*S.mutans*); and four species of oral commensal bacteria: *Streptococcus sanguinis* (*S.sang.*), *Streptococcus mitis* (*S.mitis*), *Veillonella parvula* (*Veill.*) and *Actinomyces viscosus* (*Act.*) were measured during the entire study period using an analogous qrt-PCR. Furthermore, clinical parameters, the Approximal Plaque Index (API) [42] and the modified Sulcular Bleeding Index (SBI) [43], were recorded at baseline before debridement, at day 7 and day 14. Oral brush biopsy-based cytology was chosen for the non-invasive investigation of oral epithelial dysplasia. At baseline and day 7 cellular material was collected by rotating a brush (Gynobrush Plus, Heinz Herenz) 10-times over the left and right buccal mucosa. The cells were transferred onto a microscopic slide and fixated (Merckofix, Merck).

On each visit medical health and medication updates, compliance and occurrence of adverse events were checked. For consistency and reliability purposes enrollment and all clinical interventions were performed by the same experienced periodontist in the facilities of the Department of Periodontics, Heinrich-Heine-University, Düsseldorf, Germany.

### Microbiological analysis

**Sample collection** One tooth per sextant was selected, based on the presence of biofilm during the screening visit. At screening, baseline, day 2, 4, 7 and 14 supragingival biofilm samples were obtained from the same teeth with a sterile hand instrument (Scaler 7835, Storz am Mark) and by swiping an additional cotton



► **Table 2** Ingredients RA1 verum and placebo mouth rinse.

ingredient RA1	specification	mass fractions (g)	function
RA		0.8	active ingredient
calcium-L-ascorbate	Ph. Eur. 6.0	0.1	stabilizer, antioxidant
ethanol 96%	Ph. Eur.	6.4	solubilizer
glycerol 85%	Ph. Eur. 6.0	15	solubilizer
potassium sorbate	Ph. Eur. 6.0	0.47	preservative
citric acid, anhydrous	Ph. Eur. 6.0	0.014	pH-stabilizer
peppermint aroma	Symrise AG, Holzminden, Germany	0.2	aroma
water, sterile, pyrogen free	B. Braun AG, Melsungen, Germany	ad 100	solvent
ingredient placebo	specification	mass fractions	function
calcium-L-ascorbate	Ph. Eur. 6.0	0.1	stabilizer, antioxidant
ethanol 96%	Ph. Eur.	6.4	solubilizer
glycerol 85%	Ph. Eur. 6.0	15	solubilizer
potassium sorbate	Ph. Eur. 6.0	0.47	preservative
citric acid, anhydrous	Ph. Eur. 6.0	0.045	pH-stabilizer
peppermint aroma	Symrise AG, Holzminden, Germany	0.2	aroma
plain orange aroma	Symrise AG, Holzminden, Germany	0.25	aroma
Symcolor caramel EBC30500	Symrise AG, Holzminden, Germany	0.1	EU-approved food colouring E 150 C
water, sterile, pyrogen free	B. Braun AG, Melsungen, Germany	ad 100	solvent

swab over the tongue, palate, cheeks (left and right) and floor of the mouth. The collected samples were immediately frozen at  $-80^{\circ}\text{C}$  until further analysis.

**DNA extraction and qrtPCR** DNA isolation was processed with the innuPREP DNA Mini Kit (Analytik Jena AG) according to the manufacturer's instructions. For qrtPCR the maxima SybrGreen Kit (Thermo Fisher Scientific) on CFX 96 BioRad was used. The bacteria specific primers and cycle settings are listed in Supplementary Data Table 15.

**Bacterial microbiome analysis** 16 s rRNA gene sequence analysis was performed on DNA isolated from one randomly selected subject from each of the test and control groups. Detailed information is provided in the Supporting Information.

### Cytopathological analysis

Oral brush biopsies were examined by an experienced cytopathologist at the Institute of Cytopathology (Heinrich-Heine-University, Düsseldorf, Germany) using the method of Dr. Papanicolaou and interpreted based on the Düsseldorf nomenclature [44].

### Statistical methods

**Sample size calculation** Because of the innovative character of the mouth rinse no data were available in literature on the estimated quantitative difference in *P.g.* or any other bacteria prevalence. Hence, the calculations were based on a large effect with a dichotomous distribution. To detect a 60% difference in the *P.g.* prevalence with a power of 80% and a  $p \leq 0.05$ , a minimum sam-

ple size of 15 persons per group was required. A 25% drop-out rate was considered as participants might have difficulty complying with the high number of visits within the 14-day study period [45]. Additionally, no data existed on the taste quality of the tested mouth rinses and potential effects on the compliance to adhere to the study protocol. Accordingly, a sample size of 40 participants was determined for the overall study population (20 per group).

**Outcomes** The primary outcome measure of this study was the change of the intraoral prevalence of *P.g.* between the test and control group at baseline, day 2, day 4, day 7 and day 14. Secondary outcomes included the change of the API and SBI values and the cytopathological appearance of the mucosal tissue. The change of prevalence of the 11 other oral microorganisms was considered as additional outcome parameters. Microbiome analysis of one test and one control subject was done as a safety precaution to detect for any alterations in the composition of the oral microflora.

**Statistical analysis** The statistical analysis was accomplished by IBM SPSS Statistics 21 (IBM). For a simple descriptive analysis continuous data were calculated as mean values with their standard deviation. Due to the small group size non-parametric testing methods were used. The Fisher exact test was performed to assess the association between demographic variables of the test and control group. Every comparison of the test- and the control-group regarding the clinical and microbiological outcomes was performed using the Mann-Whitney-U-test. Multiple compar-

isons of time variables were conducted by the Friedman-test and their significant data were confirmed by the post-hoc Wilcoxon rank test with a Bonferroni adjusted alpha. The level of significance was set at  $p \leq 0.05$ .

## Competing Financial Interests Statement

This study was financially supported by the European Union (ZIEL.2.NRW program) with funding to TB (grant number 300262502) and AH (grant number w1006sb014). Residual expenses were borne by intramural annual budgets from the University of Düsseldorf to TB and the University of Münster to AH. The study design, data collection and analysis, as well as the intention to publish and its completion have not been influenced by the funders.

## Supporting Information

The references for the bacteria specific primers used in the qrtPCR (see Table 1S) are listed in the Supporting Information.

The results of high throughput 16S rRNA gene sequence analysis of the oral bacterial microbiome from one control (SA120) and one test subject (SA088), at each time-point (V1–V6), are presented in the Supporting Information (see Fig. 1S, 2S).

## Contributors' Statement

S.S. conducted the clinical trial, clinical sample and data acquisition and drafted the manuscript. S.B. and A.H. manufactured the test and placebo solution and were responsible for the randomization. Sample processing and bioinformatics analysis was completed by U.P., R.W. and R.T. The conception, study design and protocol, data analysis and interpretation were performed by S.S., A.K., U.P., T.F. and partially T.B. All authors have critically revised and approved the final manuscript.

## Conflict of Interest

The authors T.B. and A.H. filed a patent application "Use of proanthocyanidins for production of an antiadhesive preparation", priority DE/14.09.09/DEA102009027696, on the 14.09.2009. However, the authors state that this application had no impact on the study protocol, its execution and evaluation and TB and AH refrained from data collection and data analysis.

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