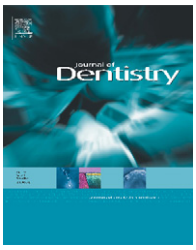
available at www.sciencedirect.com

ScienceDirect

journal homepage: www.intl.elsevierhealth.com/journals/jden



Polyphenolic beverages reduce initial bacterial adherence to enamel *in situ*

Christian Hannig [a,*], *Julia Sorg* [a], *Bettina Spitzmüller* [a], *Matthias Hannig* [b], *Ali Al-Ahmad* [a]

[a] *Department of Operative Dentistry and Periodontology, University of Freiburg, Hugstetter Str. 55, D-79106 Freiburg, Germany*
[b] *Clinic of Operative Dentistry, Periodontology and Preventive Dentistry, University Hospital, Saarland University, Building 73, D-66421 Homburg/Saar, Germany*

ARTICLE INFO

Article history:
Received 12 February 2009
Received in revised form
23 March 2009
Accepted 24 March 2009

Keywords:
Pellicle
Biofilm
In situ
Polyphenol
FISH
DAPI
Cistus

ABSTRACT

Objectives: Polyphenols are antibacterial and anti-oxidative natural agents. The present *in situ* study aimed to investigate the effect of different polyphenolic beverages on initial bacterial adherence to enamel in the oral cavity.

Methods: Initial biofilm formation was performed on bovine enamel specimens mounted buccally on individual upper jaw splints and carried by six subjects. After 1 min of pellicle formation, oral rinses with black tea, green tea, grape juice, Cistus tea or red wine were performed for 10 min. Afterwards the slabs were carried for another 19 or 109 min, respectively. Samples exposed to the oral fluids for 30 and 120 min served as controls. Following intraoral exposure, the slabs were rinsed with saline solution. The amount of adherent bacteria was determined with DAPI-staining (4′,6-diamidino-2-phenylindole) and with fluorescence-*in situ* hybridization (FISH) of eubacteria and streptococci.

Results: Rinses with all beverages reduced the amount of detectable bacteria. Lowest number of adherent bacteria was found following rinses with red wine, Cistus tea and black tea as measured with DAPI (up to 66% reduction of adherent bacteria vs. controls). Also FISH revealed significant impact of most tested beverages.

Conclusions: Rinses with certain polyphenolic beverages as well as consumption of these foodstuffs may contribute to prevention of biofilm induced diseases in the oral cavity.

1. Introduction

The two challenging diseases in dentistry, caries and periodontitis are caused by pathogenic oral biofilms.[1,2] Chlorhexidine is a well proven rinse for the biofilm management in the oral cavity, especially in acute infections or before and after oral surgery.[3,4] However, chronic adoption of chemotherapeutics such as chlorhexidine cannot be recommended as this may induce resistances of some bacterial strains as well as a general shift of the oral flora and irritations of the taste.[5,6] Accordingly, mild agents are desirable for complementary plaque control and disinfection of the oral cavity, characteristics provided by certain natural agents.[2] Polyphenols are a group of natural organic substances with two or more phenol units. They are classified either as hydrolyzable tannins such as gallic acid esters of glucose and other sugars or as phenylpropanoids like lignins or flavonoids, respectively. Polyphenols are regarded as the most abundant antioxidants in diet yielding free radical-scavenging properties.[7] Thus, these natural substances are discussed as potential candidates for chemoprevention and treatment of cancer and cardiovascular diseases, though extensive further research is required to validate their benefits.[8] Even neuroprotective properties and possible benefits on Alzheimer's disease by

* *Corresponding author*. Tel.: +49 761 270 4888; fax: +49 761 270 4762.
E-mail address: christian.hannig@uniklinik-freiburg.de (C. Hannig).
0300-5712/$ – see front matter
doi:10.1016/j.jdent.2009.03.017

Table 1 – Polyphenolic beverages used for 10 min mouth rinses in the study.

	Producer	Preparation
Red wine	Rioja 2006 (Viña Pasarela, Alfaro, Spanien)	–
Purple grape juice	Black grape juice, unfiltered (Alnatura, Bickenbach, Deutschland)	–
Cistus tea	Cistus incanus, Zistrose Bio Tee, Dr. Pandalis Naturprodukte, Glandorf, Germany	A volume of 200 ml of no longer boiling hot water was added to 2 g of dried Cistus tea, the tea was infused for 7 min
Black tea	Darjeeling (Tea Götz GmbH, Hannover, Germany)	A volume of 200 ml of no longer boiling hot water was added to 4 g of dried black tea, the tea was infused for 7 min
Green tea	Gunpowder (Alnatura, Bickenbach, Germany)	A volume of 200 ml no longer boiling hot water was added to 4 g of dried Green-tea, the tea was infused for 7 min

breakdown of beta-amyloid and direct effects on neural tissues are described in the literature.[7,9] Also anti-viral properties have been described for certain polyphenolic compounds.[10,11] Besides these special features, polyphenols are known to have antibacterial effects.[12,13] Beverages rich in polyphenols are red wine, black tea and green tea. A disadvantage of red wine is the presence of ethanol but several studies have shown up that the beneficial effects of red wine in prevention of cardiovascular diseases can be achieved with grape juice, too.[14] Another tea, rich in polyphenols besides green and black tea is Cistus which has considerable impact on the initial bacterial colonization of enamel surfaces.[15]

The adherence of micro-organisms to solid substrates in the oral cavity is governed by the pellicle, a layer formed almost immediately from the oral fluids.[16] Polyphenols seem to have a tanning effect on this proteinaceous film to become physically harder.[16,17] It is noteworthy that black tea extract was shown to decrease caries formation in hamsters[18]; and wines are active against oral streptococci.[19]

However, the impact of other polyphenolic beverages than Cistus tea on the initial bacterial adherence to enamel *in situ* has not been quantified systematically until now.

Modern fluorescence microscopic methods, i.e. DAPI-staining and FISH allow exact quantification of adherent micro-organisms.[20,21] Thus, the present study aimed to investigate the effect of consuming different common polyphenolic beverages on initial bacterial adherence *in situ* in comparison to Cistus tea as a sequel to a previous investigation.[15]

2. Methods

2.1. Subjects and specimens

Six healthy volunteers participated in the study. Visual oral examination was carried out by an experienced dentist. The subjects showed no signs of gingivitis or caries. Informed written consent had been given by the subjects about participation in the study. The study design was reviewed and approved by the Medical Ethic Committee of the Medical Association of Saarland, Germany (52/05). Cylindrical enamel slabs (diameter 5 mm, 19.63 mm^2 surface area, height 1.5 mm) were prepared from labial surfaces of bovine incisors of BSE-negative 2-year-old cattle. The surfaces were polished by wet grinding with abrasive paper (400–4000 grit). The smear layer on the slabs was removed by ultrasonication with NaOCl for 3 min.[21–23] Afterwards, the samples were disinfected in ethanol (70%) for another 3 min, washed in distilled water and stored in aqua dest. for 24 h before exposure in the oral cavity.[22]

2.2. Pellicle and initial biofilm formation, application of the polyphenolic beverages

For *in situ* pellicle formation, individual upper jaw splints were vacuum-formed from 1.5 mm thick methacrylate foils. Cavities were prepared in the buccal aspects of the splints at the sites of the premolars and the 1st molar on the left and on the right side (n = 4/splint). The slabs were fixed on the splints with polyvinyl siloxane impression material (Aquasil light body, Dentsply DeTrey, Konstanz, Germany), exposing only the surfaces of the specimens to the oral environment.

The splints were carried in the oral cavity for 1 min to allow pellicle formation on the surfaces. Afterwards, the subjects rinsed for 10 min with 200 ml of different polyphenolic beverages (Table 1). A mouthful of the certain fluids was kept in the oral cavity and swallowed or disgorged, followed by another sip, simulating slow consumption of these fluids. The temperature of the beverages was 20 °C. After the rinse, the splints remained in the oral cavity for another 19 or 109 min. In the following, the enamel slabs were immediately dismounted from the splints and thoroughly rinsed with running tap water for 5 s in order to remove non-adsorbed salivary remnants. Samples exposed to the oral fluids for 30 and 120 min without application of a beverage served as controls. One experiment was carried out per subject and day at 10.00 a.m. to ensure standardized application and a wash out of the polyphenolic beverages, one pass of the experiments was performed per subject and per beverage for 30 or 120 min each, respectively.

The 4 enamel slabs per pass and subject were tested for the amount of adherent bacteria with DAPI and FISH, each with two samples.

2.3. Total bacterial count (DAPI)

DAPI-staining was conducted as described previously.[21] 4′,6-diamidino-2-phenylindole (DAPI) stains DNA unspecifically by binding to the AT-rich regions of double stranded DNA.[24] Upon binding to DNA, the DAPI-molecule fluoresces intensely. For staining, enamel slabs were covered with 1 ml DAPI solution (Merck, Darmstadt, Germany). After 10 min the DAPI solution was removed by rinsing with distilled water. Subsequently, the enamel specimens were dried at room temperature and coated with Citifluor (Citifluor Ltd., London, UK) on a slide and

analysed by epifluorescence microscopy (Axioskop II, Zeiss, Oberkochen, Germany). The initial biofilms were analysed with 1000-fold magnification using a light filter for DAPI (BP 365, FT 395, LP 397). The number of cells observed in 10 randomized microscopic ocular grid fields per sample was counted. The area of ocular grid (0.0156 mm^2) allowed estimating the numbers of bacteria per cm^2.

2.4. *Fluorescence in situ hybridization (FISH)*

FISH was conducted according to Amman et al. and was adapted on bovine enamel slabs as described previously.[20,21,25] Initial biofilms formed on enamel slabs were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS, 1.7 mM KH_2PO_4, 5 mM Na_2HPO_4 with 0.15 M sodium chloride, pH 7.2) for 12 h at 4 °C. After fixation, all specimens were washed with phosphate-buffered saline and incubated again in a solution containing ethanol (50% in PBS, v/v). Subsequently, the specimens were washed twice with PBS, followed by incubation in a solution containing 7 mg of lysozyme (hen egg white, 105,000 U/mg, Fluka, Buchs, Switzerland) per ml of 0.1 M Tris–HCl, 5 mM EDTA (pH 7.2), for 10 min at 37 °C in order to permeabilize the bacteria. Afterwards, the samples were dehydrated with a series of ethanol washes. Specimens were then incubated with the oligonucleotide samples at a concentration of 50 ng per 20 ml of hybridization buffer (0.9 M NaCl, 20 mM Tris–HCl (pH 7.2), 25% formamide (v/v) and 0.01% sodium dodecyl sulphate (w/v)). Following probe hybridization, specimens were incubated for 15 min in wash buffer containing 20 mM Tris–HCl (pH 7.5), 5 mM EDTA, 159 mM NaCl and 0.01% sodium dodecyl sulphate (w/v). After washing, the labelled biofilms were analysed by epifluorescence microscopy (Axioskop II, Zeiss, Oberkochen, Germany) at a magnification of 1000-fold. The number of bacterial cells detected in 10 randomized microscopic ocular grid fields per specimen was counted. The size of the counting field (0.0156 mm^2) allowed calculation of the bacterial cells/cm^2. Both HPLC purified oligonucleotide probes for streptococci and eubacteria used in this study were synthesized commercially and 5′-end labelled with different fluorochromes (Thermo Electron GmbH, Ulm, Germany). EUB 338 (5′-GCTGCCTCCCGTAGGAGT-3′) was labelled with fluorescein and used to visualize the entire bacterial population within the plaque specimens. STR 405 (5′-TAGCCGTCCC-TTTCTGGT-3′) was 5′-labelled with cy3 and used to visualize oral streptococci.

2.5. *Statistics*

Statistical evaluation was performed by ANOVA followed by the Scheffé-procedure ($p < 0.05$). The software used was SPSS 16.0.

3. Results

With both methods, a significant impact of the polyphenolic beverages on the amount of detectable adherent bacteria was

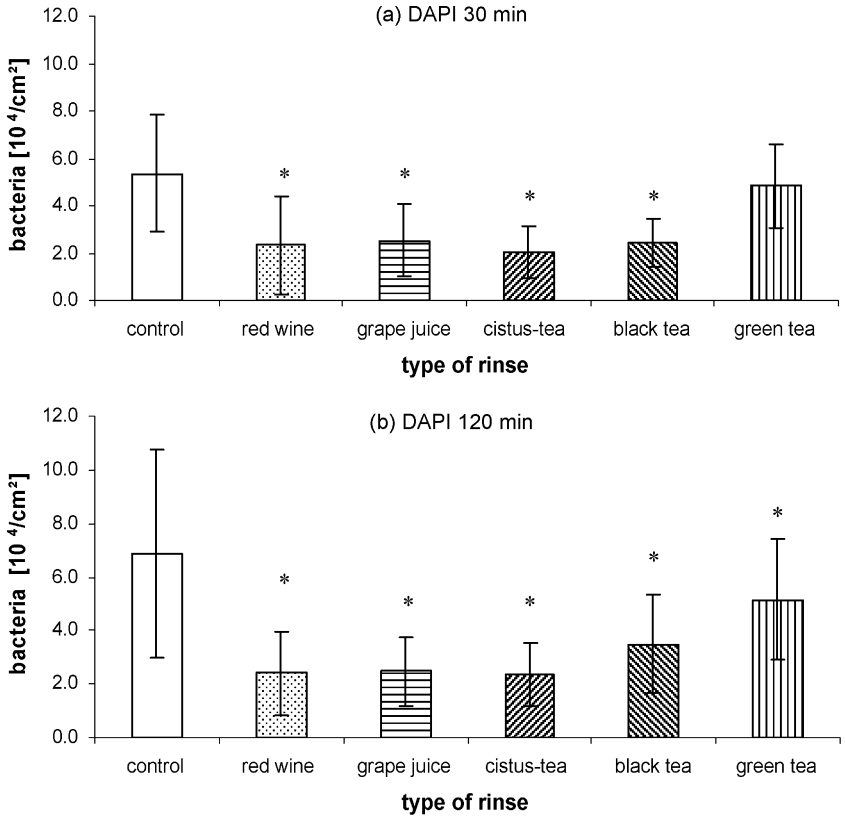


Fig. 1 – DAPI-staining after rinses with polyphenolic beverages for detection of adherent bacteria. Exposition of enamel slabs at buccal sites of the upper 1st and 2nd premolar and 1st molar for 30 and 120 min, respectively, MV ± S.D., *n* = 12 samples per subgroup (*n* = 6 subjects, 2 samples per subject, beverage and oral exposure time, respectively). Data significantly different from controls are marked (*).

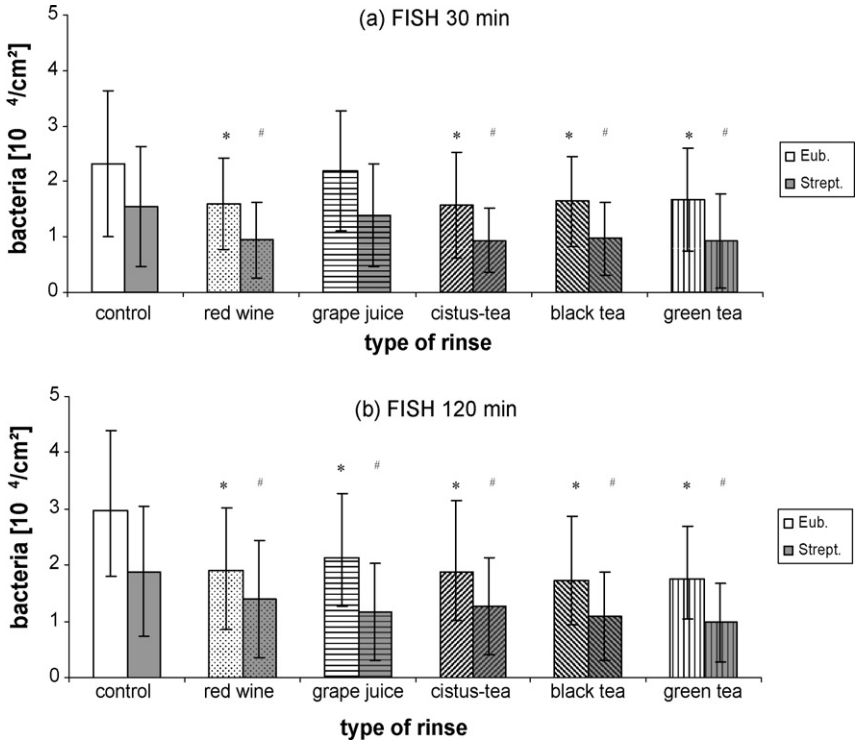


Fig. 2 – Fluorescence *in situ* hybridization (FISH) for detection of adherent eubacteria (Eub) and streptococci (Strept) after rinses with polyphenolic beverages. Exposition of enamel slabs at buccal sites of the upper 1st and 2nd premolar and 1st molar for 30 and 120 min, respectively, MV ± S.D., *n* = 12 samples per subgroup (*n* = 6 subjects, 2 samples per subject, beverage and oral exposure time). Data significantly different from controls are marked (EUB: *, STREP: #).

observed (ANOVA; FISH, DAPI: $p < 0.000$) (Figs. 1 and 2). The rinses with the different beverages had no effect on the general appearance of the bacterial aggregates as observed with CLSM (Fig. 3). Single bacteria as well as chains or monolayered aggregates of micro-organisms were detectable at the surfaces of the enamel slabs. Mainly cocci but also rods and fibrils were visible.

3.1. *DAPI*

Significantly strongest reduction of bacterial adherence was observed with Cistus tea, red wine and grape juice, whereas green tea was of lowest efficacy, especially after the 30 min period (Fig. 1). After 30 min a reduction of up to 50% vs. controls was observed with Cistus tea, red wine and grape juice; after 120 min even up to 66% less bacteria were detected after application of these beverages as compared with unrinsed samples. However, the oral exposure time had no effect on the amount of micro-organisms detectable on control specimens.

3.2. *FISH*

With FISH less pronounced effects were recorded as compared with the DAPI-data (Fig. 2). However, a significant reduction of adherent bacteria was observed with all beverages except of grape juice after 30 min (Fig. 2). After 120 min all samples rinsed with polyphenolic beverages exposed fewer eubacteria and streptococci than the control specimens.

Also with FISH no significant impact of the oral exposure time on the amount of detectable micro-organisms was found.

4. Discussion

Biofilm formation on the tooth-surface is determined considerably by the process of initial bacterial adherence to the pellicle layer.[21,26–28] Mechanisms like co-adhesion of bacteria as well as the interactions with the pellicle components contribute to this process.[27,28] Thus, samples exposed to the oral fluids for 30 or 120 min, respectively, were evaluated. Furthermore, the study focussed on buccal sites as in a previous reference study.[21] DAPI and FISH are accepted methods for the visualisation and quantification of micro-organisms in the adherent state. Both methods had been adapted to the investigation of initial biofilms formed *in situ* in the oral cavity. Thereby, the use of bovine enamel slabs is a well established approach for standardized plaque and pellicle formation.[20,21,29] The polyphenolic compounds were consumed over a 10 min period simulating a slow drinking and rinsing which is not uncommon for these beverages. As in previous studies, a considerable intraindividual and interindividual variability was recorded which seems to be characteristic for initial bioadhesion in the oral cavity. However, with FISH and especially with DAPI-staining, a significant reduction of adherent bacteria was recorded. This was particularly true for Cistus tea, red wine and black tea, respectively. However, the effects observed with FISH were

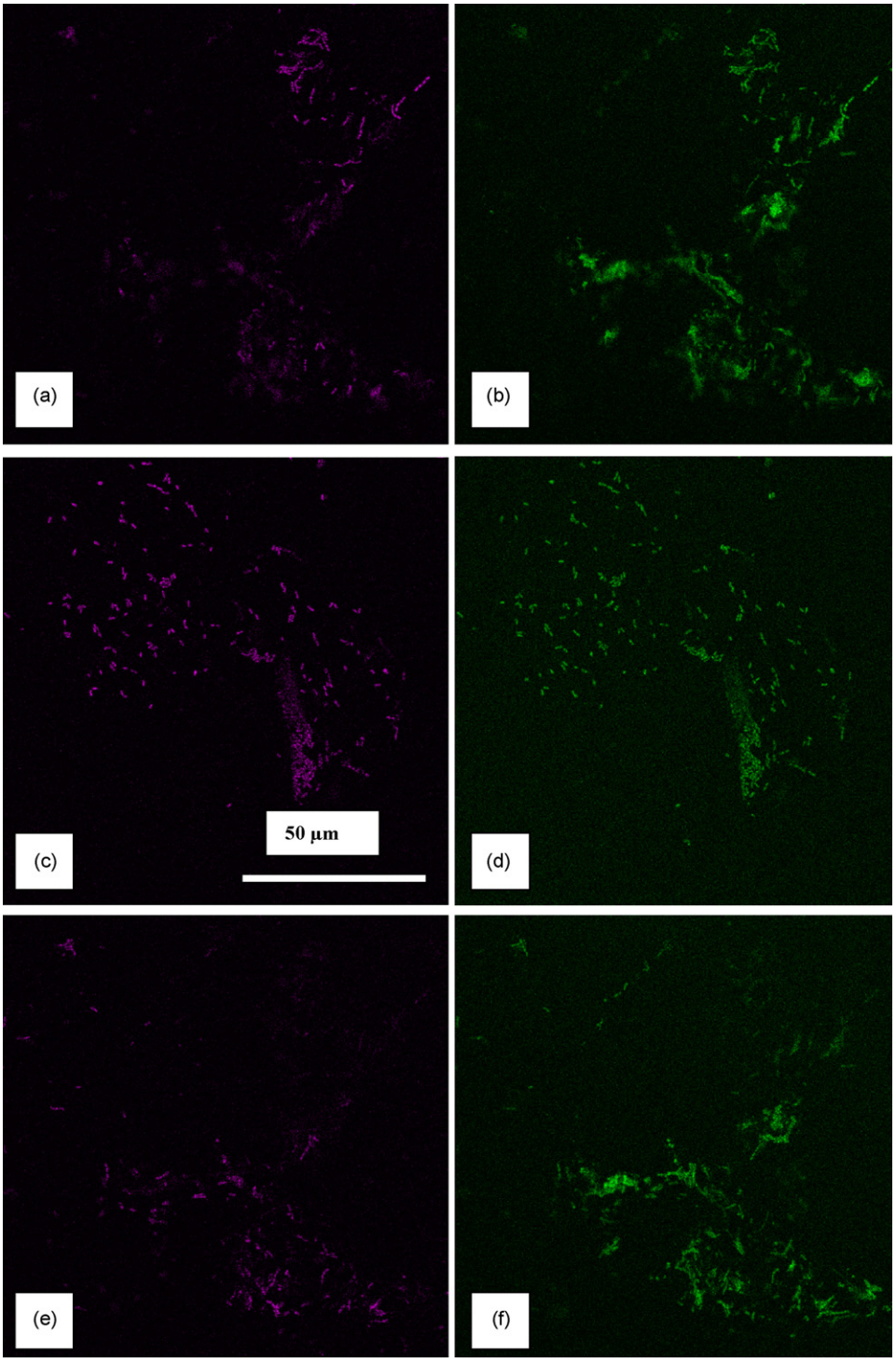


Fig. 3 – Visualization of adherent bacteria by FISH after 120 min exposure to the oral fluids. Figures (a and b: controls) as well as (c and d: green tea) and (e and f: grape juice) depict the same sections; eubacteria (green) and streptococci (magenta). Please note single bacteria as well as chains and aggregates of micro-organisms. Original magnification: 1000-fold. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article).

not as pronounced. The bacteria detected with FISH are assumed to represent the major part of viable micro-organisms,[20] due to the relative abundance of ribosomes in sound bacteria required for this method.[25,30] However, the staining of cells that died just before fixation cannot be excluded. In a 2-day-old oral biofilm from different subjects, 77% of the bacteria were shown to be viable using a vital fluorescence assay, [31] whereas approximately 57% of the bacteria adhering during the first 120 min yielded viability.[21] This corresponds well to the present data; irrespective of the

rinses, more bacteria were detected with DAPI based on a general DNA-staining than with FISH.

The reduction of bacterial adherence to the pellicle layer and the antibacterial effects may be induced by several different mechanisms.[32] Relevant polyphenolic compounds are catechins, and flavonal-O-glycosides such as myricetin-galactoside, myricetin-rhamnoside and quercetin-glucoside.[32] Among them, catechins are regarded as the most relevant molecules for antibacterial effects.[33] A mixture of catechins inhibited the adherence of streptococci on hydroxyapatite in a previous study.[34] Polyphenolic compounds are also capable of reducing the viability of these bacteria *in vitro*.[13]

The inhibition of salivary enzymes involved in the carbohydrate anabolism and catabolism of glucolytic bacteria might also be a complementary factor. Amylase as well as glucosyltransferases are inhibited by polyphenols.[35,36] A reduction of glucan-formation by glucosyltransferase means also less binding sites for specific bacterial adhesion to the tooth surface.[37] Furthermore, other bacterial enzymes are inhibited by polyphenols.[38] Nevertheless, a previous *in situ* study showed up that Cistus tea had no impact on enzyme activities immobilised in the acquired pellicle.[15]

Besides these influences on enzymes, polyphenols have tanning effects on the pellicle and its proteinaceous components.[16,39,40] This might mask or denature the functional groups of receptor proteins aggravating interactions with bacteria. Typical pellicle components such as histatin or proline rich proteins interact with polyphenols thereby forming complexes.[41,42] This explains their astringent properties.[42]

These mechanisms do not only occur directly at the tooth surface, but also affect bacteria and enzymes in the saliva or on the soft tissues, respectively. This is of great interest as the upper surface of the tongue is regarded as some kind of reservoir for bacterial (re)-colonization of the tooth surfaces after oral hygiene procedures. The amount of bacteria present at the tongue after rinses with polyphenolic beverages requires further investigation.

All in all, polyphenolic rinses do not eliminate bacterial adherence to the tooth surface but some of them reduce it considerably. This can be regarded as an additive positive side effect of these beverages which are regarded helpful in the prevention of cardiovascular diseases.[8] However, from a cariological point of view, the characteristics of the certain polyphenolic foods have to be analysed in detail. Unfiltered purple grape juice might reduce bacterial adherence but due to the erosive pH of any fruit juices[43] and due to the fructose content it cannot be recommended as a prophylactic regimen in dentistry.[44] Also the adoption of red wine is difficult because of the alcoholic content especially for patients suffering from Xerostomia or for children. Green tea was not as effective as Cistus tea or black tea regarding the DAPI-data. It may be postulated that some kind of fermentation is necessary for the full establishment of the antibacterial properties of black tea. Also the thein of black tea might be problematic though it contains considerable amounts of fluoride. Last but not least, Cistus tea was included in the study. This tea does not contain any serious components and is therefore recommendable even for children or for patients with Sjögreńs syndrome.[10,15,45]

5. Conclusions

- Some polyphenolic beverages reduce initial bacterial adherence to enamel *in situ* beside their well-known antibacterial properties.
- Consumption of certain polyphenolic beverages as well as rinses might contribute to prevention of biofilm induced diseases in the oral cavity.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgements

The study was supported in part by a grant from the DFG (Deutsche Forschungsgemeinschaft, #HA 5192/1-2 #HA 2718/3-3).

REFERENCES

1. Marsh P. Dental plaque: biological significance of a biofilm and community life-style. *Journal of Clinical Periodontology* 2005;**32**:7–15.
2. Marsh P. Antimicrobial strategies in the prevention of dental caries. *Caries Research* 1993;**27**:72–6.
3. Mandel ID. Chemotherapeutic agents for controlling plaque and gingivitis. *Journal of Clinical Periodontology* 1988;**15**:488–98.
4. Twetman S. Antimicrobials in future caries control? A review with special reference to chlorhexidine treatment. *Caries Research* 2004;**38**:223–9.
5. Deng DM, ten Cate JM, Crielaard W. The adaptive response of *Streptococcus mutans* towards oral care products: involvement of the ClpP serine protease. *European Journal of Oral Sciences* 2007;**115**:363–70.
6. Paraskevas S, Rosema NA, Versteeg P, Van der Velden U, Van der Weijden GA. Chlorine dioxide and chlorhexidine mouthrinses compared in a 3-day plaque accumulation model. *Journal of Periodontology* 2008;**79**:1395–400.
7. Singh M, Arseneault M, Sanderson T, Murthy V, Ramassamy C. Challenges for research on polyphenols from foods in Alzheimer's disease: bioavailability, metabolism, and cellular and molecular mechanisms. *Journal of Agricultural and Food Chemistry* 2008;**56**:4855–73.
8. Giovannini C, Scazzocchio B, Vari R, Santangelo C, D'Archivio M, Masella R. Apoptosis in cancer and atherosclerosis: polyphenol activities. *Annali Dell'istituto Superiore di Sanita* 2007;**43**:406–16.
9. Baxter RA. Anti-aging properties of resveratrol: review and report of a potent new antioxidant skin care formulation. *Cosmetic Dermatology* 2008;**7**:2–7.
10. Sassi AB, Harzallah-Skhiri F, Bourgougnon N, Aouni M. Antiviral activity of some Tunisian medicinal plants against Herpes simplex virus type 1. *Natural Product Research* 2008;**22**:53–65.
11. Droebner K, Ehrhardt C, Poetter A, Ludwig S, Planz O. CYSTUS052 a polyphenol-rich plant extract, exerts anti-influenza virus activity in mice. *Antiviral Research* 2007;**76**:1–10.
12. Yoshida T, Hatano T, Ito H. Chemistry and function of vegetable polyphenols with high molecular weights. *Biofactors* 2000;**13**:121–5.

13. Smullen J, Koutsou GA, Foster HA, Zumbe A, Storey DM. The antibacterial activity of plant extracts containing polyphenols against *Streptococcus mutans*. *Caries Research* 2007;**41**:342–9.
14. Folts JD. Potential health benefits from the flavonoids in grape products on vascular disease. *Advances in Experimental Medicine and Biology* 2002;**505**:95–111.
15. Hannig C, Spitzmüller B, Al-Ahmad A, Hannig M. Effects of Cistus-tea on bacterial colonization and enzyme activities of the *in situ* pellicle. *Journal of Dentistry* 2008;**36**:540–5.
16. Hannig M, Joiner A. The structure, function and properties of the acquired pellicle. in: Duckworth R (Ed.). Monographs in oral sciences, vol. 19;2006:p. 29–64.
17. Joiner A, Linden JA, Hutchings IM. The scratch hardness of *in vitro* formed pellicle. *Journal of Dental Research* 2000;**79**:1202.
18. Linke HA, LeGeros RZ. Black tea extract and dental caries formation in hamsters. *International Journal of Food Sciences and Nutrition* 2003;**54**:89–95.
19. Daglia M, Papetti A, Grisoli P, Aceti C, Dacarro C, Gazzani G. Antibacterial activity of red and white wine against oral streptococci. *Journal of Agricultural and Food Chemistry* 2007;**55**:5038–42.
20. Al-Ahmad A, Wunder A, Auschill TM, Follo M, Braun G, Hellwig E. The *in vivo* dynamics of *Streptococcus* spp., *Actinomyces naeslundii*, *Fusobacterium nucleatum* and *Veillonella* spp. in dental plaque biofilm as analysed by five-colour multiplex fluorescence *in situ* hybridisation. *Journal of Medical Microbiology* 2007;**56**:681–7.
21. Hannig C, Hannig M, Rehmer O, Braun G, Hellwig E, Al-Ahmad A. Fluorescence microscopic visualization and quantification of initial bacterial colonization on enamel *in situ*. *Archives of Oral Biology* 2007;**52**:1048–56.
22. Deimling D, Breschi L, Hoth-Hannig W, Ruggeri A, Hannig C, Nekrashevych Y, *et al*. Electron microscopic detection of salivary alpha-amylase in the pellicle formed *in situ*. *European Journal of Oral Sciences* 2004;**112**:503–9.
23. Hannig M, Khanafer AK, Hoth-Hannig W, Al-Marrawi F, Acil Y. Transmission electron microscopy comparison of methods for collecting *in situ* formed enamel pellicle. *Clinical Oral Investigations* 2005;**9**:30–7.
24. Schwartz T, Hoffmann S, Obst U. Formation of natural biofilms during chlorine dioxide and u.v. disinfection in a public drinking water distribution system. *Journal of Applied Microbiology* 2003;**95**:591–601.
25. Amann RI, Ludwig W, Schleifer KH. Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. *Microbiological Reviews* 1995;**59**:143–69.
26. Hannig M, Hannig C. Does a biofilm free of bacteria, exist *in situ*? *Journal de Parodontologie and Implantogie Orale* 2007;**26**:187–200.
27. Marsh P, Martin M. Oral microbiology. Oxford: Wright; 1999.
28. Marsh P, Bradshaw DJ. Dental plaque as a biofilm. *Journal of Industrial Microbiology and Biotechnology* 1995;**15**:169–75.
29. Hannig M. Transmission electron microscopy of early plaque formation on dental materials *in vivo*. *European Journal of Oral Sciences* 1999;**107**:55–64.
30. Amann RI, *In situ* identification of microorganisms by whole cell hybridization with rRNA-targeted nucleic acid probes.Akkermans ADL, van Elsas JD, de Bruijin FJ, editors. *Molecular microbial ecology manual*, vol. 3.3.6. Dordrecht, the Netherlands: Kluwer Publishers; 1995. p. 1–15.
31. Arweiler NB, Hellwig E, Sculean A, Hein N, Auschill TM. Individual vitality pattern of *in situ* dental biofilms at different locations in the oral cavity. *Caries Research* 2004;**38**:442–7.
32. Chung KT, Wong TY, Wei CI, Huang YW, Lin Y. Tannins and human health: a review. *Critical Reviews in Food Science and Nutrition* 1998;**38**:421–64.
33. Hamilton-Miller JM. Anti-cariogenic properties of tea (*Camellia sinensis*). *Journal of Medical Microbiology* 2001;**50**:299–302.
34. Otake S, Makimura M, Kuroki T, Nishihara Y, Hirasawa M. Anticaries effects of polyphenolic compounds from Japanese green tea. *Caries Research* 1991;**25**:438–43.
35. Kandra L, Gyemant G, Zajacz A, Batta G. Inhibitory effects of tannin on human salivary alpha-amylase. *Biochemical and Biophysical Research Communications* 2004;**319**:1265–71.
36. Yanagida A, Kanda T, Tanabe M, Matsudaira F, Oliveira Cordeiro JG. Inhibitory effects of apple polyphenols and related compounds on cariogenic factors of mutans streptococci. *Journal of Agricultural and Food Chemistry* 2000;**48**:5666–71.
37. Schilling KM, Bowen WH. Glucans synthesized *in situ* in experimental salivary pellicle function as specific binding sites for *Streptococcus mutans*. *Infection and Immunity* 1992;**60**:284–95.
38. Zhang F, Luo SY, Ye YB, Zhao WH, Sun XG, Wang ZQ, *et al*. The antibacterial efficacy of aceraceous plant may be related to inhibition of bacterial beta-ketoacyl-ACP reductase (FabG). *Biotechnology and Applied Biochemistry* 2008;**51**:73–8.
39. Joiner A, Muller D, Elofsson UM, Arnebrant T. Ellipsometry analysis of the *in vitro* adsorption of tea polyphenols onto salivary pellicles. *European Journal of Oral Sciences* 2004;**112**:510–5.
40. Joiner A, Muller D, Elofsson UM, Malmsten M, Arnebrant T. Adsorption from black tea and red wine onto *in vitro* salivary pellicles studied by ellipsometry. *European Journal of Oral Sciences* 2003;**111**:417–22.
41. Yan Q, Bennick A. Identification of histatins as tannin-binding proteins in human saliva. *Biochemical Journal* 1995;**311**:341–7.
42. Baxter NJ, Lilley TH, Haslam E, Williamson MP. Multiple interactions between polyphenols and a salivary proline-rich protein repeat result in complexation and precipitation. *Biochemistry* 1997;**36**:5566–77.
43. Lussi A, Jaeggi T, Zero D. The role of diet in the aetiology of dental erosion. *Caries Research* 2004;**38**:34–44.
44. Sreebny LM. Sugar and human dental caries. *World review of nutrition and dietetics* 1982;**40**:19–65.
45. Ehrhardt C, Hrincius ER, Korte V, Mazur I, Droebner K, Poetter A, *et al*. A polyphenol rich plant extract, CYSTUS052, exerts anti influenza virus activity in cell culture without toxic side effects or the tendency to induce viral resistance. *Antiviral Research* 2007;**76**:38–47.