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Isolation of red wine components with anti-adhesion and anti-biofilm activity against *Streptococcus mutans*

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ABSTRACT

Red wine is a widely consumed beverage with multiple beneficial effects on human health. In the present paper, the anticaries properties of red wine were studied *in vitro* and *ex vivo*. Our *in vitro* findings shows that dealcoholised red wine, besides exerting antibacterial activity, strongly interferes with *Streptococcus mutans* adhesion to saliva-coated hydroxyapatite (sHA) beads, promotes its detachment from sHA, and powerfully inhibits *in vitro* biofilm formation. The main components responsible for such activities were found to be proanthocyanidins. The ability of red wine to inhibit *ex vivo S. mutans* biofilm formation on the occlusal surface of natural human teeth also was demonstrated.

Our data indicates that protection of the oral cavity from the cariogenic action of *S. mutans* may be another beneficial effect of the moderate consumption of red wine.

1. Introduction

The health benefits of moderate wine consumption are well documented, and have been associated with increased longevity and a diminished risk of cardiovascular and neurological diseases (Pinder & Sander, 2004; Saremi & Arora, 2008; Ullah & Khan, 2008). The protective effects of wine may be related to the combined effects of ethanol and non-alcoholic components, such as polyphenols. The latter are responsible for its organoleptic properties (e.g., colour, astringency, bitterness, and aroma) and are associated with beneficial physiological effects, such as free radical scavenging, intracellular metal chelation, inhibition of transcription factors, and enzyme modulation ability (Rodrigo & Bosco, 2006; Singh, Arseneault, Sanderson, Murthy, & Ramassamy, 2008).

Wine contains a large number of phenolic substances; ca. 200 mg of phenols are found in a glass of red wine (Waterhaouse, 2002). Phenolics include non-flavonoids, such as hydroxycinnamates, hydroxybenzoates, stilbenes, and flavonoids, like flavonols, anthocyanins, and flavan-3-ol. Most phenolic compounds, especially those found in red wine, derive from the condensation of flavan-3-ol into oligomers (proanthocyanidins) and polymers (condensed tannins). The phenolic composition of wine is affected by grape variety and by factors influencing berry development, such as soil, geographical location, and weather conditions. Wine-making techniques also play an important role through phenol extraction from grapes, as do time of maceration and fermentation in contact with grape skins and seeds, pressing, maturation, fining and bottle ageing (Monagas, Bartolomé, & Gomez-Cordoves, 2005).

Over the past three decades several studies have described the anticaries effect of phenols extracted from foods and beverages (Daglia et al., 2002; Duarte et al., 2006; Gregoire, Singh, Vorsa, & Koo, 2007; Matsumoto, Hamada, & Ooshima, 2003; Okamoto, Leung, Ansai, Sugimoto, & Maeda, 2003; Okamoto et al., 2004; Yamanaka et al., 2007; Yanagida, Kanda, Tanabe, Matsudaira, & Oliveira Cordeiro, 2000). Caries is a multifactor infectious disease involving interactions among diet constituents, cariogenic bacteria and the tooth surface. Caries prevention interventions aim mainly at eradicating cariogenic streptococci, particularly *Streptococcus mutans*, which is widely regarded as the primary microbial cause of tooth decay in humans. Given the role of bacterial glucans both in promoting the accumulation of streptococci on the tooth surface and in favoring bacterial aggregation in a polyspecies consortium (biofilm), known as dental plaque (Loesche, 1986), a promising prophylactic approach is to inhibit glucan production from dietary carbohydrates (mainly sucrose) by bacterial glucosyltransferases (GTFs). Another approach-based on the facts that bacteria adhere to the dental surface by mechanisms that include hydrophobic,

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electrostatic and specific ligand-receptor interactions, and that adherence, in turn, is a prerequisite for biofilm formation-consists in blocking/modifying *S. mutans* surface sites involved in adhesion, thus reducing the efficiency of interactions with tooth surfaces. Since acid production by *S. mutans* contributes to tooth enamel demineralisation, leading to caries formation, another preventive approach may be to reduce the ability of acidogenic bacteria to produce and tolerate acid, through inhibition of proton translocating F-ATPase, a bacterial enzyme that protects *S. mutans* against environmental acid stress via regulation of pH homeostasis (Marsh & Martin, 1999; Socransky, Haffajee, Cugini, Smith, & Kent, 1998). Recently, red wine grape extracts and the fermented byproducts of winemaking were found to inhibit GTFs B and C at very low concentrations, and to reduce *S. mutans* acidogenicity (Thimothe, Bonsi, Padilla-Zakour, & Koo, 2007). Previous work by our group also showed that both red and white wine exert *in vitro* antibacterial activity against several oral streptococci and induce postcontact effects against *S. mutans*. The organic acids deriving from grapes, or formed during malolactic fermentation, were found to be responsible for such activities (Daglia et al., 2007). The above considerations prompted us to extend the investigation to other wine properties to confirm the anticaries activity of wine. To do this we investigated the ability of the beverage and its fractionated components to affect *S. mutans* adhesion to and detachment from hydroxyapatite (HA) beads and to inhibit biofilm formation on microtiter plates. Anti-biofilm activity was also tested in an *ex vivo* model.

2. Materials and methods

2.1. Chemicals

Potassium phosphate buffer (PB), sodium hydroxide, ethyl acetate, methanol, ethanol, diethylether, hydrochloric acid, sodium hypochlorite, and acetone were purchased from Sigma–Aldrich (Milan, Italy). [Methyl-^{3}H]thymidine was obtained from Amersham Biosciences Europe GmbH (Cologno Monzese, Italy). Spheroidal HA beads were purchased from Fin-ceramica (Faenza, Italy). Brain Heart Infusion Broth (BHIB) and Brain Heart Infusion Agar (BHIA) were purchased from LAB M, Ltd. (Lancashire, UK).

2.2. Wine sample and dealcoholisation

An Italian red wine, Valpolicella Classico DOC Superiore, vintage: 2003 (pH 3.56, alcohol 13.5%), was purchased from Cantina Sociale della Valpolicella, Negrar, Verona. Following dealcoholisation by vacuum concentration (50 mbar) at 30 °C; the dealcoholised wine (DRW) was filtered through Millipore membranes of cellulose acetate/cellulose nitrate mixed esters (0.45 μm) (Millipore Corp., Billerica, MA). The dry residue was determined by evaporation according to the AOAC Official Method of Analysis (1995). DRW was tested for pH value and the ability to affect *S. mutans* adhesion to and detachment from HA beads and to form biofilm both on microtiter plates at different concentrations and on natural teeth. All experiments were performed in triplicate.

2.3. Solid phase extraction (SPE)

SPE was performed according to Sun, Leandro, da Silva, and Spranger (1998), with some modifications (Daglia et al., 2007). A 5 mL aliquot of DRW was concentrated to dryness in a rotary evaporator at <30 °C. The residue was dissolved in 20 mL PB (pH 7.0) and adjusted to pH 7.0 with NaOH solution. Two C18 Sep-Pak cartridges (Waters, Milford, MA) connected in series were conditioned with methanol (10 mL), distilled water (2 × 10 mL), and PB (pH 7.0, 10 mL). Samples were then passed through the cartridges at a flow rate ⩽2 mL min^{-1}. The polar substances were eluted first, with 10 mL PB, pH 7.0 (SPE F1). After the cartridges were dried with N_2, simultaneous elution of monomeric and oligomeric flavan-3-ols (SPE F2 and SPE F3) was obtained with 25 mL ethyl acetate, followed by elution of polymeric proanthocyanidins (SPE F4) with 15 mL methanol. The ethyl acetate fraction was taken to dryness under vacuum, redissolved in 3 mL PB, and finally redeposited onto the same conditioned cartridges. Cartridges were dried with N_2 and monomers (SPE F2) were separated from oligomers (SPE F3) by sequential elution with 25 mL diethyl ether and 15 mL methanol. Sample fractionation was performed in duplicate. The four SPE fractions were concentrated to eliminate organic solvents and freeze-dried. Dry matter was determined and the residue dissolved in 2.5 mL Millipore grade water (2× concentration), and finally tested for pH value and the ability to affect *S. mutans* adhesion to and detachment from HA beads and to form biofilm on microtiter plates at different concentrations. All experiments were performed in triplicate.

2.4. Gel filtration chromatography (GFC)

GFC was performed according to Sun, Leandro, de Freitas, and Spranger (2006), with some modifications. All experiments were performed using a 1100 HPLC system (Agilent, Waldbronn, Germany) equipped with a gradient quaternary pump, and a diode array detector (DAD) system. The Agilent Chemstation software was used for HPLC system control and data processing. A Superformance Universal glass cartridge system (300 × 10 mm) (Merck, Darmstadt, Germany) was used for GFC separation of the SPE4 fraction. The stationary phase was Toyopearl® HW-40F with exclusion limits of 100–10,000 Da (TOSOH BIOSCIENCE GmbH, Stuttgart, Germany). The elution was carried out at a flow rate of 0.5 mL min^{-1}, with an injected volume of 1 mL. Chromatograms were acquired at 280 and 520 nm. To obtain the GFC1 fraction the mobile phase was Millipore grade water for 60 min and methanol/hydrochloric acid (at the concentration of 0.01%, v/v) (7/3, v/v) for 60 min; to obtain the GFC2 fraction the mobile phase was Millipore grade water/acetone (8/2, v/v). GFC fractions were reconstituted to 1/2 of the initial volume (2× concentration) by rotary evaporation of the SPE4 fraction and different concentrations were tested for their ability to inhibit *S. mutans* adhesion to HA beads and biofilm formation on microtiter plates. All experiments were performed in triplicate.

2.5. Bacterial strains, growth media, and chemicals

S. mutans 9102 and *S. mutans* ATCC 25175 were used throughout the study. Bacteria were cultured in BHIB and BHIA at 37 °C in the presence of 5% CO_2; when sucrose dependent adhesion was analysed, 0.1 M sucrose (final concentration) was added to the culture medium. For the radiolabelling procedure streptococci were grown in THB containing 10 μCi [methyl-^{3}H]thymidine (25 Ci mmol^{-1}) mL^{-1}. Cells were harvested in the mid-exponential phase by centrifugation (5000*g* for 15 min at 4 °C) and washed twice with an equal volume of 10 mM PB, pH 7.0. Cell labelling efficiency varied among strains and ranged from 200 to 1400 cells/cpm.

2.6. Evaluation of DRW and DRW fraction minimal inhibitory concentration (MIC)

DRW and DRW fractions were serially diluted (1/1, v/v) in 96 well microtiter plates in BHIB, which was the medium used in the biofilm assay. The microtiter plates were inoculated with *S. mutans* 9102 and *S. mutans* ATCC 25175 cells (1 × 105 cells mL^{-1}, final concentration) and subsequently incubated for 24 h at 37 °C

in 5% CO_2 atmosphere. The MIC was defined as the lowest concentration of the fractions that inhibited visible bacterial growth.

2.7. Bacterial adsorption to HA beads

Fifty mg aliquots of spheroidal HA beads (grain size 250–875 μm) were washed three times in 1 mM PB, pH 7.0, in glass tubes and then equilibrated for 2 h in the same buffer.

Beads were allowed to settle for 30–60 s and the supernatant was removed by aspiration. Beads were then treated with 200 μL saliva that was collected, clarified and used undiluted, as described previously (Tarsi, Muzzarelli, Guzmàn, & Pruzzo, 1997). DRW (or DRW fractions) at different concentrations and the radiolabelled bacterial suspensions (final concentration: 5–7 × 107 cells mL^{-1}) were added simultaneously to saliva-coated HA (sHA) beads in polypropylene microfuge tubes and incubated at room temperature (RT) on a Wheaton Mini Drum Roller (Wolf Laboratories Ltd., Pocklington, UK) at 20 rpm, as described above (experimental approach A). In a different set of experiments (experimental approach B), beads were pretreated with the beverage (or with DRW fractions) and then incubated at RT on the Wheaton Mini Drum Roller at 20 rpm. After 1 h, the beads were washed, the radiolabelled bacterial suspension was added to the beads at a final concentration of 5–7 × 107 cells mL^{-1}, and the mixture was incubated as above. Control samples without the beverage (or the DRW fractions) were included in all treatments. After 1 h incubation the beads were collected by centrifugation (200*g*, 5 min, 4 °C), washed four times with 10 mM PB to remove non-adherent bacteria, and transferred to PICO-FLUORTM 15 scintillation fluid (Packard Instruments Company Inc., Ill.). Radioactivity was assayed with an L5 1801 scintillation counter (Beckman Instruments, Fullerton, CA). Cell labelling was used to measure the number of bacteria adsorbed to the sHA beads. The inhibitory activity (AIA%) of the materials was gauged by comparing DRW (or DRW fraction) treated samples to the respective untreated controls (100%). Controls for bacterial settling due to aggregation were also included; the amount of settled bacteria was always <1% of the inoculum. Experiments were run in triplicate and were performed at least twice.

2.8. Bacterial detachment from sHA beads

The ability of different concentrations of DRW (or DRW fractions) to detach *S. mutans* cells from sHA beads was determined as described by Tarsi, Corbin, Pruzzo, and Muzzarelli (1998). All the experiments were run in triplicate. The radiolabelled bacterial suspension (1 mL) was added to the sHA beads, which were collected by centrifugation (200*g*, 5 min, 4 °C) after 1 h incubation and washed four times with 10 mM PB, to remove non-adherent bacteria. A separate sample was used to assess total sHA bound radioactivity. DRW (or DRW fractions) were then added to bacteria treated beads; the mixtures were centrifuged as described above, respectively, at time 0 and after 1 and 2 h incubation at RT on the Wheaton Mini Drum Roller at 20 rpm. Labelled bacteria in the supernatants were counted. Untreated control samples were also included. The ratio of detached/total bead bound bacteria was then determined; results are expressed as percentage of bacterial detachment (BD%). Experiments, run in triplicate, were performed at least twice.

2.9. Biofilm formation assay

The ability to form stable biofilms was assessed by growing *S. mutans* cells in 96 well, flat bottom microtiter plates (Greiner Bio-one Cellstar, Frickenhausen, Germany) as described previously (Wen, Suntharaligham, Cvitkovitch, & Burne, 2005) Briefly, overnight cultures of *S. mutans* were transferred to prewarmed BHIB and grown at 37 °C in an atmosphere containing 5% CO_2 to an OD600 of ca. 0.5. Cultures were diluted 1:100 in fresh BHIB, with or without sucrose; 200 μL aliquots containing different concentrations of DRW (or DRW fractions) were inoculated into the wells of the plates. Negative controls, i.e., wells containing uninoculated growth medium, and positive controls, i.e., wells containing inoculated growth medium without DRW (or DRW fractions), were included. Plates were incubated at 37 °C in 5% CO_2 atmosphere for 48 h. For biofilm quantification, the plates were slowly immersed in water and shaken to remove any remaining planktonic bacteria or loosely bound cells. After doing this twice, the plates were blotted on paper towels and air dried. Adherent bacteria were stained with 50 μL of 0.1% crystal violet for 15 min at RT; the plates were then slowly immersed in water twice, to rinse the wells. The bound dye was extracted from stained cells by adding 200 μL of ethanol/acetone (8/2, v/v). Biofilm formation was then quantified by measuring the absorbance of the solution at 540 nm. Biofilm inhibitory activity (BIA%) was evaluated as a proportion of untreated controls (100%).

2.10. Biofilm formation on natural teeth

Natural teeth from extractions performed at the Dental Clinic of the University of Verona, preserved in sterile saline solution at 4 °C, were treated with sodium hypochlorite for 20 min at RT and extensively washed with sterile saline solution. They were then placed in bacterial cultures which contained or did not contain DRW. The supernatants were changed every 2 days. After 7 days the amount of biofilm produced was assessed with a stereomicroscope (Leica Zoom 2000, Leica Microsystems Inc., Educational and Analytical Division, Buffalo, New York, NY, USA) at 20× magnification.

2.11. Statistical analysis

The adsorption efficiency of DRW treated and untreated samples was analysed on the same day. Differences were calculated using Student's *t* test and the χ^2 test.

3. Results and discussion

3.1. DRW effects on S. mutans adherence to and detachment from sHA beads

A commercial Italian red wine, Valpolicella Classico DOC Superiore, was dealcoholised before testing, to prevent interference from ethanol. Dealcoholised red wine (DRW) dry matter and pH were 19.07 mg mL^{-1} (1× concentration) and 3.505, respectively. Two different approaches were adopted to assess the effects of DRW on sucrose dependent and independent bacterial adhesion to sHA beads: under experimental approach A, streptococci were added to sHA in the presence of DRW; under approach B, the beads were pretreated with DRW before adding the bacteria, to mask bacterial receptors on sHA. As illustrated in Fig. 1, both experimental approaches resulted in dose dependent inhibition of *S. mutans* adsorption to saliva-coated beads, irrespective of the presence of sucrose. For both the ATCC 25175 and the 9102 strain the lowest DRW concentration causing a significant decrease in bacterial adhesion (AIA% ranging from 17 ± 1% to 26 ± 2%) was 4.77 mg dry matter mL^{-1}, corresponding to a 0.25× concentration. These findings suggest that the anti-adhesion properties of DRW may be due to the masking of saliva treated HA receptors for bacterial ligands as well as to the masking and/or modification of bacterial adhesions. The ability of DRW to promote bacterial detachment from sHA was studied by adding DRW to radiolabelled bacteria adhering to the beads, in the presence and absence of sucrose.

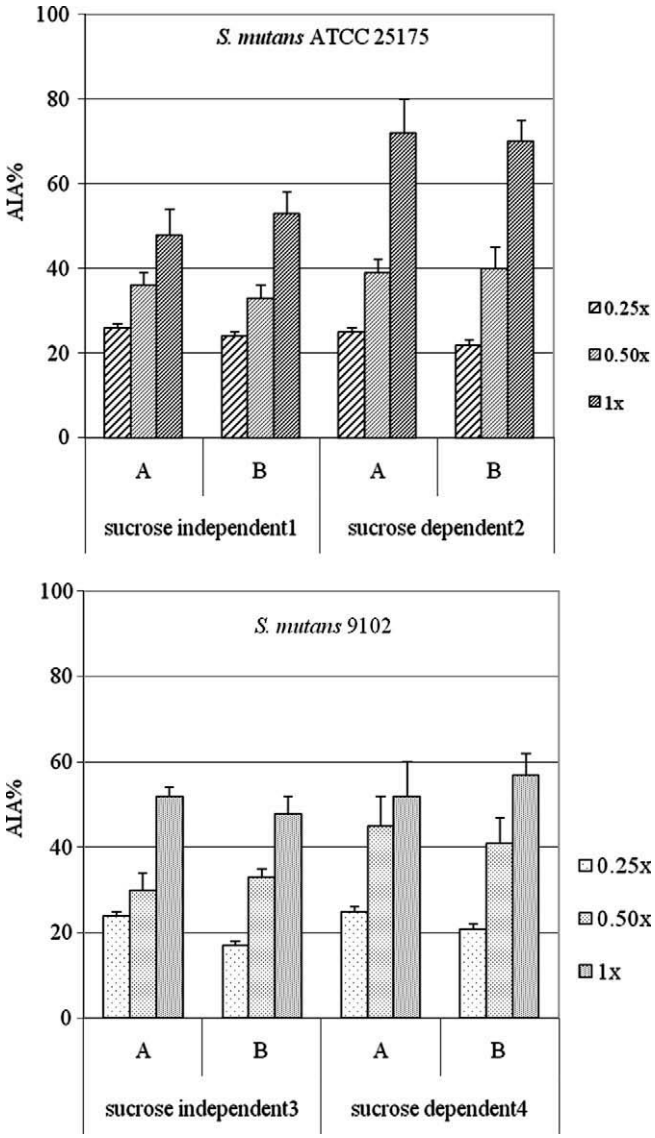

Fig. 1. Adhesion inhibitory activity (AIA%) of *S. mutans* to saliva coated HA beads by different DRW concentrations added simultaneously (A) or as pretreatment (B). [1]control = 6.6 ± 0.3; [2]control = 9.0 ± 1.0; [3]control = 10.9 ± 1.0; [4]control = 12.8 ± 1.7.

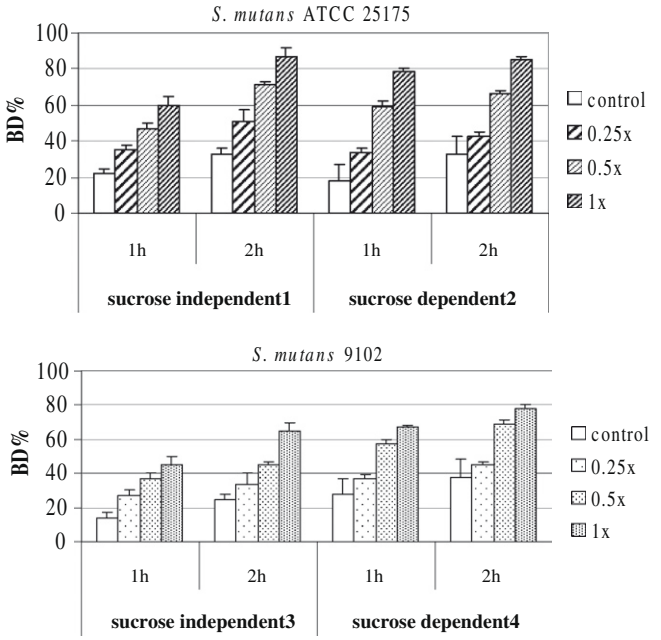

Fig. 2. Bacterial detachment (BD%) from saliva coated HA beads by different DRW concentrations after 1h and 2h incubation. [1] × 10^6bacteria/50 mg HA + SD = 6.0 ± 0.43; [2] = 9.7 ± 0.4; [3] = 7.8 ± 0.4; [4] = 11.3 ± 1.3.

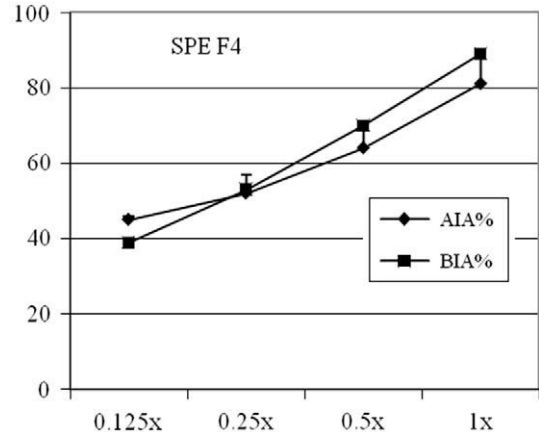

Fig. 3. Adhesion inhibitory activity (AIA%) of *S. mutans* ATCC 25175 to saliva coated HA beads and biofilm inhibitory activity (BIA%) by different SPE F4 concentrations in presence of sucrose.

After 1 and 2 h incubations, BD% was determined 250 by measuring supernatant radioactivity. Compared to control samples DRW caused a significant increase in *S. mutans* ATCC 25175 and 9102 detachment from the beads even at the lowest concentration tested (4.77 mg dry matter mL^{-1}), as in the case of the adhesion test (Fig. 2).

3.2. Effects of DRW fractions on S. mutans adhesion to and detachment from sHA beads

The above findings prompted isolation of the compound(s) responsible for the anti-adhesion properties of DRW. To this purpose, DRW was fractionated by SPE based on the degree of polymerisation of flavan-3-ol. DRW was fractionated into four fractions: the first (SPE F1), eluted with PB, contained organic acids, residual sugars, and the other polar water soluble wine components. Monomeric and oligomeric flavan-3-ols were eluted simultaneously with ethyl acetate (SPE F2 and F3). The last fraction (SPE F4), containing polymeric flavan-3-ols, was eluted with methanol. Further separation of the SPE F2 and F3 fractions by another SPE with sequential elution yielded monomeric flavan-3-ols (SPE F2) using diethyl ether, and oligomeric compounds (SPE F3) using methanol. Restoration of the original volume enabled SPE fraction constituents testing at the concentrations found in the beverage (1×). Dry matter and pH values were, respectively, 4.00 mg mL^{-1} and 6.920 (SPE F1); 7.11 mg mL^{-1} and 7.152 (SPE F2); 3.02 mg mL^{-1} and 6.918 (SPE F3), and 5.06 mg mL^{-1} and 6.846 (SPE F4).

In the adhesion experiments with the SPE fractions (1× concentration) conducted in the presence of sucrose according to procedure A, adhesion inhibitory activity was detected only for SPE F4 (AIA% = 81.5 ± 5% and 79.0 ± 1% with *S. mutans* ATCC 25175 and *S. mutans* 9102, respectively). The fraction exerted a significant inhibitory effect until the lowest concentration tested, 0.062 mg dry matter mL^{-1}, corresponding to a 0.125× concentration (Fig. 3). Interestingly the AIA% of SPE F4 was much higher than that of DRW (AIA% SPE F4 0.125× = 12 47.0 ± 1% vs AIA% DRW 0.25× = 25.0 ± 1%). These data

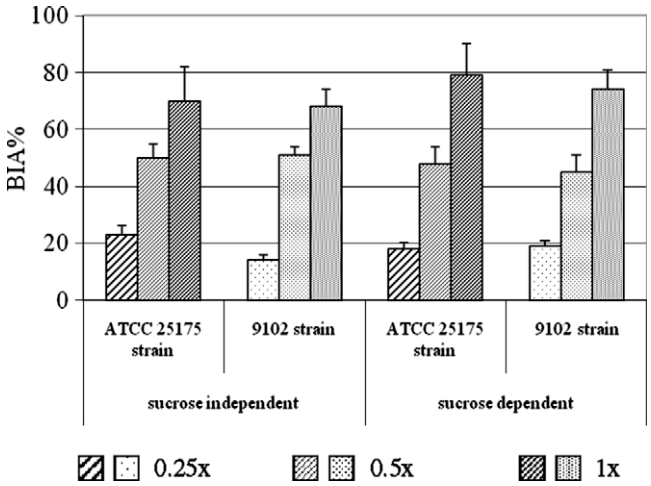

Fig. 4. Biofilm inhibitory activity (BIA%) of *S. mutans* ATCC 25175 and *S. mutans* 9102 by different DRW concentrations in the presence and absence of sucrose.

suggest that the anti-adhesion properties of the active DRW components may be inhibited by interactions occurring between them and/or with other wine components.

In the detachment assays SPE F4 was again the sole fraction endowed with anti-adhesion activity, albeit it was very low. In fact, after 1 and 2 h incubation at 1× concentration detached vs adherent ATCC 25175 bacteria were 13 ± 1% and 21 ± 2%, respectively, with the SPE F4 fraction, and 6 ± 1% and 8 ± 0%, respectively, in the control sample. Similar results were obtained with *S. mutans* 9102 (data not shown). To identify the compounds responsible for these effects the SPE F4 fraction, which contains red pigments and proanthocyanidins (polymeric flavan-3-ol), was subjected to GFC. Separation of wine pigments from proanthocyanidins was achieved by elution and recorded at 520 and 280 nm. The red pigments (GFC1) were first eluted with water and then with methanol:hydrochloric acid. When no more coloured compounds were eluted from the column, the second fraction, containing proanthocyanidins (GFC2), was recovered by elution with water:acetone. Each GFC fraction was taken to dryness under vacuum, brought to the initial DRW volume (1× concentration) and analysed for anti-adhesion activity against *S. mutans* ATCC 25175 in the presence of sucrose. The results showed that both fractions were active at 1× concentration, and that GFC1 and GFC2 both reduced bacterial adhesion to HA beads by 36.0 ± 5% and 48.0 ± 7%, respectively. Interestingly, the sum of the AIA% values of GFC1 and GFC2 was almost equivalent to that of the whole SPE F4 fraction (AIA% DRW = 81.5 ± 5%).

Neither GFC1 nor GFC2 showed a significant ability to detach bacteria from HA beads, suggesting that the ability of DRW and SPE F4 to promote bacterial detachment is to be ascribed to a combination of compounds, or that the initially active compounds were inactivated by GFC fractionation.

3.3. Effects of DRW and DRW fractions on biofilm formation

The above data prompted evaluation of the effects of DRW and DRW fractions on biofilm formation, another *S. mutans* property linked to its cariogenic potential. The biofilm forming ability of the ATCC 25175 and 9102 strain was studied using the microtiter plate assay. After 24 h incubation at 37 °C in the presence and absence of sucrose, DRW showed a significant BIA% up to the final concentration of 4.77 mg dry matter mL^{-1}, corresponding to a 0.25× concentration (Fig. 4), that was also the lowest active concentration for *S. mutans* adherence to and detachment from sHA beads.

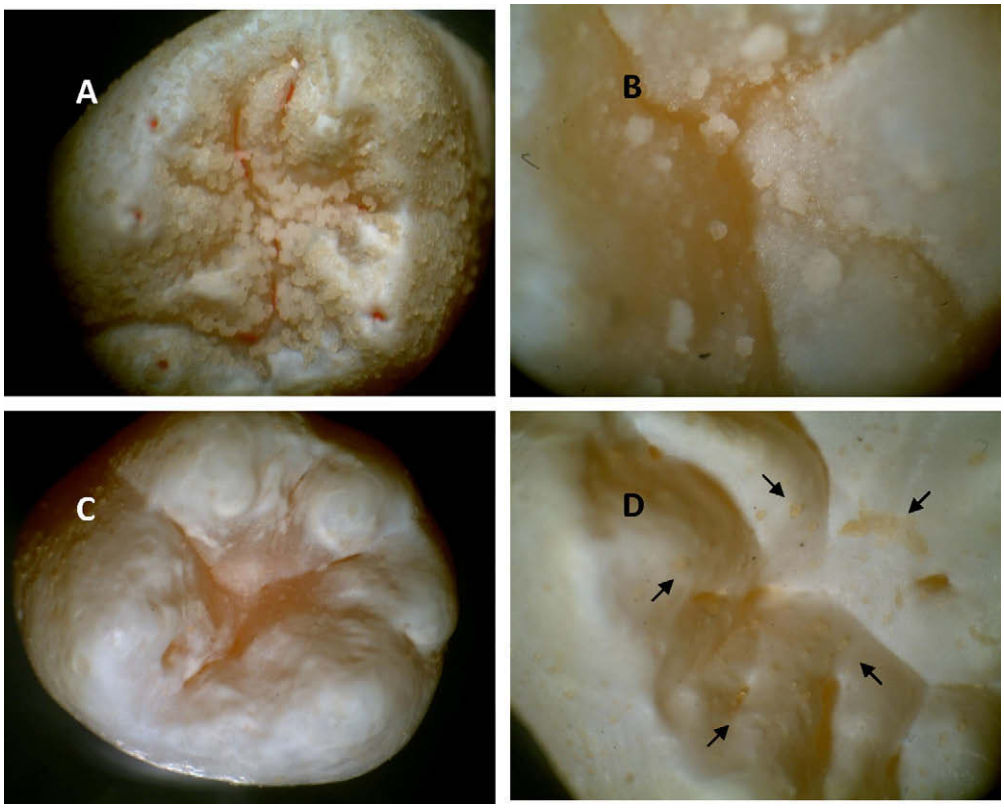

Fig. 5. Biofilm formation by *S. mutans* on the crown of a natural tooth after 7 days (A) and (B) at higher magnification, and in the presence of DRW (0.5 × concentration) (C) and (D) at higher magnification.

Then the ability of SPE fractions to inhibit biofilm formation in the presence of sucrose by *S. mutans* ATCC 25175 was studied. At the highest concentrations tested, corresponding to that of the beverage (1×), SPE fractions F1, F2, and F3 showed no significant inhibition of biofilm formation (data not shown), whereas SPE F4 activity was very high (BIA% SPE F4 = 89 ± 4%), and close to the activity of the whole beverage (BIA% DRW = 79 ± 3%). Testing of different SPE F4 concentrations showed a linear dose–response relationship ($r = 0.9812$) until a 0.125× concentration (0.062 mg dry matter mL^{-1}) (Fig. 3). As noted in the adhesion test, its BIA% was much higher than that of DRW (BIA% SPE F4 0.125× = 39.0 ± 1%, BIA% DRW 0.25× = 18.0 ± 1%), again suggesting a negative synergism among wine components. Both GFC fractions deriving from SPE F4 inhibited biofilm formation, GFC1 showing activity (BIA% = 67 ± 3%) and GFC2 very high activity (BIA% = 98 ± 5%). Testing of different GFC2 concentrations showed a linear dose response relation ($r = 0.9826$) down to the lowest concentration tested, corresponding to a 0.125× concentration. Therefore each GFC fraction obtained from the sole active SPE fraction, SPE F4, corresponding to 26.5% of DRW dry matter, possessed very high anti-biofilm activity. These results also showed that the components of each fraction exerted a negative influence on the other's activity.

To establish whether the anti-biofilm action of DRW and its fractions was due to their antibacterial activity their MICs against *S. mutans* ATCC 25175 were obtained in the seven experimental conditions used for the microtiter plate assay. The MIC of DRW was 19.07 mg dry matter mL^{-1}, corresponding to that of the whole beverage. Of the SPE fractions only SPE F4 was active, its MIC of 2.53 mg dry matter mL^{-1} corresponding to a 0.5× DRW concentration. Also in this case SPE F4 had a higher activity than DRW. Of the GFC fractions, only GFC2 showed antibacterial activity, to a concentration of 0.5×. These data suggest that the anti-biofilm activity of DRW and of the SPE and GFC fractions depends both on their antibacterial activity and, at subMIC values, on interference with the adhesion mechanism. However, an inhibitory effect on matrix exopolysaccharide production should also be considered.

3.4. Effects of DRW on biofilm formation on natural teeth

An *ex vivo* study conducted to confirm the anticaries activity of DRW assessed its ability to inhibit *S. mutans* biofilm formation on the occlusal surface of natural human teeth that were obtained from dental extraction procedures. After 7 days growth in the presence of sucrose, *S. mutans* ATCC 25175 was seen to cover tooth surfaces and to give rise to bacterial piles organised as a sort of biofilm. Teeth cultured in the presence of DRW (final concentration: 2.38 mg dry matter mL^{-1}) exhibited rare bacterial microcolonies (Fig. 5).

4. Conclusions

Our findings show that DRW, besides exerting antibacterial activity (Daglia et al., 2007), strongly interferes with *S. mutans* adhesion to substrates such as sHA beads, natural human teeth and plastic, promotes its detachment from sHA, and powerfully inhibits *in vitro* biofilm formation. DRW component fractionation on the basis of polarity showed that proanthocyanidins were the components most involved in the anti-adhesion, anti-biofilm and antibacterial activity of wine. Red pigments (found in GFC1), though efficient in inhibiting *S. mutans* adhesion and biofilm formation at 1× concentration, had no antibacterial activity. Experiments are in progress in our laboratories to chemically characterise the active components of GFC1 and GFC2. Such purified components will enable detection of bacterial and/or salivary target(s) and elucidation of the mechanisms of action.

In conclusion, this study supports our previous findings that wine may have anticaries properties, and recognises its proantocyanidin fraction as the most active component. Protection of the oral cavity from the cariogenic action of *S. mutans* may therefore be added to the health benefits of moderate red wine consumption previously ascribed only to its polyphenols.

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