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Red Wine and Oenological Extracts Display Antimicrobial Effects in an Oral Bacteria Biofilm Model

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ABSTRACT: The antimicrobial effects of red wine and its inherent components on oral microbiota were studied by using a 5-species biofilm model of the supragingival plaque that includes *Actinomyces oris*, *Fusobacterium nucleatum*, *Streptococcus oralis*, *Streptococcus mutans* and *Veillonella dispar*. Microbiological analysis (CFU counting and confocal laser scanning microscopy) of the biofilms after the application of red wine, dealcoholized red wine, and red wine extract solutions spiked or not with grape seed and inactive dry yeast extracts showed that the solutions spiked with seed extract were effective against *F. nucleatum*, *S. oralis* and *A. oris*. Also, red wine and dealcoholized wine had an antimicrobial effect against *F. nucleatum* and *S. oralis*. Additional experiments showed almost complete and early degradation of flavan-3-ol precursors [(+)-catechin and procyanidin B2] when incubating biofilms with the red wine extract. To our knowledge, this is the first study of antimicrobial properties of wine in an oral biofilm model.

KEYWORDS: *wine, polyphenols, oral bacteria biofilm, antimicrobials*

INTRODUCTION

The oral cavity is an enormously complex habitat with several hundred commensal microbial species colonizing it, and furthermore, it is unique in the human body in possessing nonshedding surfaces, the teeth, allowing microorganisms to adhere to the surface of teeth for long periods of time, embedded in a self-produced matrix of extracellular polymeric substances,[1] and thus leading to extensive biofilm formation, dental plaque,[2] which is more resistant than planktonic cells to mechanical stress or antibiotic treatment.[3] The microorganisms of dental plaque live with one another in a commensal or mutualistic symbiotic relationship, allowing a mixture of aerobic and anaerobic bacteria to live in the same environment. Some of these oral bacteria, such as streptococci or lactobacilli, are able to produce high levels of organic acids following fermentation of dietary sugars. Acids released from dental plaque lead to demineralization of the tooth surface and consequently to dental caries, periodontal disease or tooth loss,[4] which are the most prevalent oral diseases in humans, affecting up to 60−90% of the world population.[5]

Even using mechanical removal, dental biofilms cannot be eliminated completely. Antimicrobial agents are complementarily used to control dental plaque.[6−8] Until now, several substances have been tested for the control of oral biofilms, including essential oils, amine fluoride, triclosan, etc., but one of the most widely used and effective antibiofilm agents is chlorhexidine.[9] However, chlorhexidine has been associated with some secondary effects, namely the reduction of human taste perception and the pigmentation of oral tissues, which limits its application. Therefore, the search for new antimicrobials has arisen, and natural products are preferable due to the lack of secondary effects and, therefore, the potential for long-term usage in the oral cavity.

The inherent matrix of the biofilm, such as extracellular polymeric substances that reduce penetration of antimicrobial agents and the presence of persistent cells surviving at low metabolic rates, contributes to the widely described phenomenon of reduced sensitivity to antimicrobial agents.[10] Because of this, biofilm models including bacteria and fungi from different species, have proven both useful and reliable in predicting *in vivo* efficacy of antimicrobials. In this sense, most experimental models for short-term studies involve a solid surface for the adhesion of bacteria.[11]

Although there is substantial literature reporting the antimicrobial properties of phenolic compounds or polyphenols against bacteria isolates,[12−14] information about their effect on oral pathogens is still scarce.[15] Studies carried out with tea and cranberry polyphenols have shown an inhibitory effect on biofilm formation by oral pathogens such as *Streptococcus mutans*, *Streptococcus oralis*, *Streptococcus sobrinus* and *Porphyromonas gingivalis*.[16] Grapes and wines are good dietary sources of polyphenolic compounds, including hydroxybenzoic and hydroxycinnamic acids, phenolic alcohols, flavan-3-ol monomers, oligomeric and polymeric procyanidins, flavonols, stilbenes and anthocyanins (only present in red varieties).[17] Recently, it has been found that wine and grape phenolic extracts, as well as pomace phenolic extracts, were able to inhibit the growth of different *Streptococcus* spp. strains associated with dental caries.[18,19]

On the other hand, interactions between wine phenolics and oral microbiota can also include a possible bacterial catabolism

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of wine phenolics into less complex phenolic metabolite structures, as seems to happen with flavonol glycosides.[15] With regard to anthocyanins, their degradation in human saliva at 37 °C has been described, being structure-dependent, largely mediated by oral microbiota, and partially suppressed after oral rinsing with antibacterial chlorhexidine.[8]

With the final aim of seeking natural products that could be used in oral hygiene and to ascertain interactions between wine components and oral microbiota, in this study the antimicrobial effects of red wine and dealcoholized red wine were investigated using a biofilm model of the supragingival plaque that integrates five bacteria species commonly associated with oral disease. A wine phenolic extract (Provinols), especially rich in anthocyanins, was also tested using the same model, and in both the absence and presence of other enological extracts from grape seeds (Vitaflavan) and yeast (inactive dry yeast, IDY). Additional experiments were carried out to determine any possible phenolic metabolism during the formation of the bacterial biofilm.

MATERIALS AND METHODS

Red Wines. The red wine used in this study was a young red wine (var. Pinot Noir, vintage 2010), kindly provided by Bodegas Miguel Torres S.A. (Catalonia, Spain). The wine was elaborated following the winery's own winemaking procedures and was selected because of its relatively high phenolic content: total polyphenols = 1758 mg of gallic acid equiv/L, total anthocyanins = 447 mg of malvidin-3-glucoside/L, and total catechins = 1612 mg of (+)-catechin/L. The main individual phenolic compounds found in this wine included anthocyanins, flavan-3-ols, flavonols, alcohols, stilbenes and hydroxycinnamic acids[20] (Table 1).

For the preparation of dealcoholized red wine, ethanol was removed using a rotary evaporator and then distilled water was added until the original volume was reached.

Oenological Extracts. A wine extract, Provinols, was kindly supplied by Safic-Alcan Especialidades S.A.U. (Barcelona, Spain). A grape seed extract, Vitaflavan, was kindly provided by Piriou (Les Dérivés Resiniques & Terpéniques S.A., France). The total phenolic content of the extracts was 474 mg of gallic acid equiv/g for Provinols and 629 mg of gallic acid equiv/g for Vitaflavan. The main phenolic compounds identified in both extracts are reported in Table 1. Also, two inactive dry yeast (IDY) commercial preparations (*Saccharomyces cerevisiae*), IDY 1 and IDY 2, rich in mannoproteins, amino acids and peptides, respectively, were kindly provided by Lallemand S.A. (Blagnac, France) and Agrovin S.A. (Alcázar de San Juan, Ciudad Real, Spain).

The wine extract was dissolved in distilled water containing 2.5% DMSO (v/v), at a concentration of 1.6 g/L. The wine extract solution was fortified in grape seed polyphenols by adding 2.5 g of grape seed extract to 100 mL of the wine solution. Also, the wine extract solution was enriched in wine matrix components (mainly polysaccharides and nitrogen compounds) by adding the IDY preparations to the wine extract solution at a final concentration of 0.4 g/L.

Bacterial Strains and Culture (Growth) Conditions. *Actinomyces oris* OMZ 745, *Fusobacterium nucleatum* OMZ 598, *Streptococcus oralis* OMZ 607, *Streptococcus mutans* UA159 (OMZ 918) and *Veillonella dispar* ATCC 17748^T (OMZ 493) were obtained from the culture collection of the Institute of Oral Biology, University of Zürich. Prior to the experiment, precultures were prepared by transferring the strains on Columbia Blood Agar plates and incubating them for 96 h at 37 °C under anaerobic conditions. After this time, the strains were transferred from the Columbia Blood Agar plates to broth cultures (1 × 9 mL of modified fluid universal medium (mFUM) + 0.3% glucose) (OMZ 493 + 1% sodium lactate) and incubated overnight at 37 °C. After incubation, 200 μL of bacteria from each working culture was individually inoculated in 5 mL of fresh mFUM and incubated at 37 °C anaerobically (7 h maximum). In order to obtain an inoculum

Table 1. Main Phenolic Compounds in Wine and Extracts

	wine (mg/L)[20]	wine extract (mg/g)[34]	grape seed extract (mg/g)[35]
	Benzoic Acids		
Gallic acid	27.30 ± 0.20	1.06 ± 0.05	9.11 ± 0.01
Protocatechuic acid	3.88 ± 0.01	*n.a*[a]	*n.a*[a]
3-*O*-Methylgallic acid	1.06 ± 0.06	*n.a*[a]	*n.a*[a]
4-Hydroxybenzoic acid	0.57 ± 0.01	*n.a*[a]	*n.a*[a]
Vanillic acid	1.85 ± 0.03	*n.a*[a]	*n.a*[a]
Syringic acid	2.30 ± 0.13	*n.a*[a]	*n.a*[a]
Benzoic acid	1.14 ± 0.06	*n.a*[a]	*n.a*[a]
Salicylic acid	0.21 ± 0.01	*n.a*[a]	*n.a*[a]
	Phenols		
Phloroglucinol	0.33 ± 0.03	*n.a*[a].	*n.a*[a]
Tyrosol	31.40 ± 1.40	18.90 ± 1.30	*n.a*[a]
Dihidroxyphenyl propan-2-ol	0.30 ± 0.04	*n.a*[a]	*n.a*[a]
	Cinnamic Acids		
Caffeic acid	6.97 ± 0.26	*n.a*[a].	*n.a*[a]
p-Coumaric acid	1.39 ± 0.02	*n.a*[a].	*n.a*[a]
Ferulic acid	0.22 ± 0.02	*n.a*[a].	*n.a*[a]
Coutaric acid	8.64 ± 0.01	2.00 ± 0.12	*n.a*[a]
Caftaric acid	4.98 ± 0.33	0.19 ± 0.07	*n.a*[a]
	Stilbenes		
Resveratrol	7.12 ± 0.29	0.43 ± 0.02	*n.a*[a]
Resveratrol-3-*O*-glucoside	*n.a*[a]	9.17 ± 0.17	*n.a*[a]
	Flavan-3-ols and Others		
(+)-Catechin	51.60 ± 1.70	9.90 ± 0.32	74.60 ± 0.09
(−)-Epicatechin	34.90 ± 2.90	6.87 ± 0.15	67.70 ± 0.75
(−)-Epicatechin-3-*O*-gallate	*n.a*[a]	0.23 ± 0.02	26.20 ± 0.41
Procyanidin B1	79.10 ± 0.90	11.10 ± 0.10	61.00 ± 1.42
Procyanidin B2	44.70 ± 0.60	4.69 ± 0.10	45.10 ± 0.95
B2-3-*O*-gallate	*n.a*[a]	0.03 ± 0.01	1.80 ± 0.06
B2-3′-*O*-gallate	*n.a*[a]	0.03 ± 0.00	1.61 ± 0.01
Procyanidin B3	16.00 ± 1.00	1.23 ± 0.02	20.40 ± 0.33
Procyanidin B4	12.90 ± 0.30	0.83 ± 0.02	15.00 ± 0.13
Procyanidin B5	2.67 ± 0.01	*n.a*[a]	*n.a*[a]
Procyanidin B7	5.75 ± 0.15	*n.a*[a].	*n.a*[a]
Procyanidin C1	14.00 ± 0.40	1.07 ± 0.04	7.07 ± 0.08
Other trimers	7.96 ± 1.05	1.24 ± 0.09	6.81 ± 0.06 (t2)
	Flavonols		
Quercetin	1.92 ± 0.01	22.40 ± 0.60	*n.a*[a]
Myricetin	0.70 ± 0.03	2.55 ± 0.07	*n.a*[a]
Kaempherol	*n.d*[b]	0.04 ± 0.01	*n.a*[a]
Quercetin-3-*O*-glucoside	*n.a*[a]	0.14 ± 0.02	*n.a*[a]
Quercetin-3-*O*-galactoside	*n.a*[a]	0.11 ± 0.01	*n.a*[a]
	Anthocyanins		
Delphinidin-3-*O*-glucoside	2.58 ± 0.11	0.57 ± 0.012	*n.a*[a]
Cyanidin-3-*O*-glucoside	0.76 ± 0.04	0.27 ± 0.01	*n.a*[a]
Petunidin-3-*O*-glucoside	4.06 ± 0.13	1.47 ± 0.03	*n.a*[a]
Peonidin-3-*O*-glucoside	18.90 ± 2.00	1.78 ± 0.01	*n.a*[a]
Malvidin-3-*O*-glucoside	36.70 ± 3.40	9.01 ± 0.50	*n.a*[a]

[a]Not analyzed. [b]Not detected.

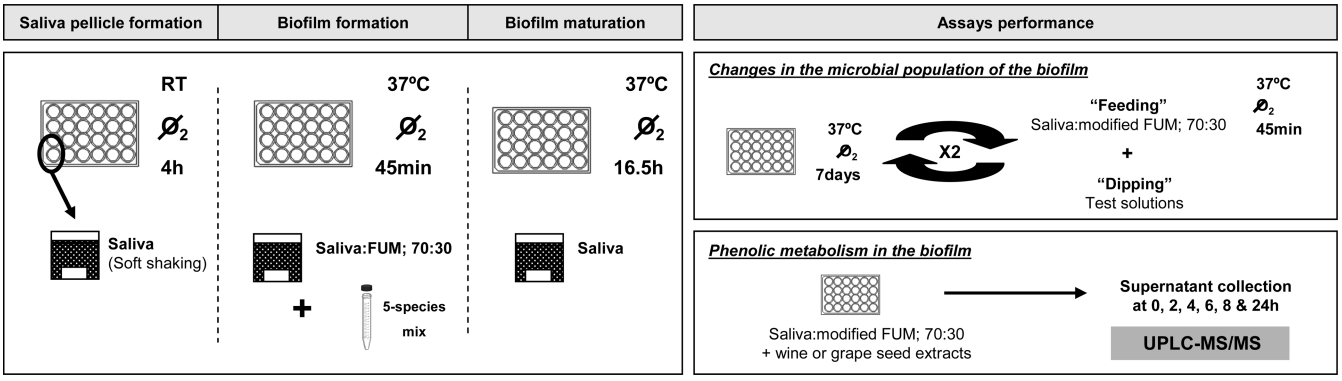

Figure 1. Biofilm formation/maturation and assays diagram.

containing cultures in the exponential growth phase of approximately 10^7 CFU/mL, a microbial suspension with equal volumes and densities of each strain was prepared.

Saliva Processing. Saliva was collected from five volunteers and processed according to the protocol of Guggenheim et al.[21] Briefly, whole unstimulated saliva was collected for 1 h each morning, over several days, at least 1.5 h after eating, drinking, or teeth cleaning. Saliva samples were collected in sterile 50 mL polypropylene tubes, chilled in an ice bath or frozen at −20 °C. After 500 mL of saliva had been collected, it was pooled and centrifuged (30 min, 4 °C, 27,000*g*); the supernatant was pasteurized (60 °C, 30 min) and recentrifuged in sterile tubes. The resulting supernatant was stored in sterile 50 mL polypropylene tubes at −80 °C. The efficiency of the process was assessed by plating the processed saliva samples onto CBA agar; after 72 h at 37 °C, no CFUs were observed on the incubated plates. A sterile 1:1 dilution in H_2O + 25% physiological NaCl was used for the biofilm formation and throughout the experimentation.

In Vitro Biofilm Experiments. Figure 1 shows a sequence chart regarding the biofilm formation prior to assays for determining changes in the microbial population of the biofilm and for assessing phenolic metabolism in the biofilm.

Biofilm Formation. Biofilms were grown using the slightly modified protocol described by Guggenheim et al.[21] and Thurnheer et al.[22] In brief, the 5-species biofilms were grown in 24-well polystyrene cell-culture plates on hydroxyapatite (HA) discs of 9 mm Ø (Clarkson Chromatography Products, South Williamsport, USA) previously preconditioned in 800 μL of whole unstimulated pooled saliva (as described in the previous section) during 4 h at room temperature, with shaking (95 rpm) in order to promote pellicle formation. To initiate the biofilm formation, the discs were covered for 45 min with 1.6 mL of a mixture comprising 30% saliva, 70% mFUM and 200 μL of the bacterial inoculum described above. mFUM corresponds to a well-established tryptone yeast-based broth medium designated as FUM[23] and modified by supplementing 67 mM Sørensen's buffer (final pH 7.2). The carbohydrate concentration in mFUM was 0.3% (w/v) and consisted of glucose for the first 16 h and from then on of a 1:1 (w/w) mixture of glucose and sucrose.

After this first incubation, discs were subjected to three consecutive 1 min dip-washes in 2 mL 0.9% NaCl to remove growth medium and free-floating cells but not microorganisms adhering firmly to the HA discs. Then, they were incubated anaerobically for 16.5 h at 37 °C in preconditioned and processed saliva to form the biofilm (Figure 1).

Assay for Determining Changes in the Microbial Population of the Biofilm. Once the biofilm was formed, discs were maintained in a 24-well plate with preconditioned and processed saliva in anaerobic conditions for 7 days. Twice a day, and with 7 h of difference in between, discs were "fed" by immersing them into a preconditioned fresh growth medium (30% saliva, 70% mFUM (v/v) containing 0.15% glucose and 0.15% sucrose) for 45 min, at 37 °C, under anaerobic conditions, After each "feeding", discs were dipped in the different test solutions (1 mL) for 2 min and while being gently shaken by hand. After this time, the discs were dipped once in the preconditioned-processed saliva in order to clean any remains of the test solutions. Immediately after, discs were returned to the "old" 24-well plate with preconditioned and processed saliva and incubated anaerobically until the next "feeding" (Figure 1). After 7 days, biofilms were either stained for confocal laser scanning microscopy (see below) or harvested, at room temperature, in 1 mL of 0.9% NaCl by scratching with a special odontological instrument. Cell viability was tested using a Live/Dead BacLight Viability Kit (Molecular Probes Inc.) The total CFU, streptococci and all taxa were assessed by anaerobic culture (37 °C) using selective (Mitis Salivarious for *Streptococcus oralis* and *Streptococcus mutans*; Fastidious Anaerobe Agar for *Fusobacterium nucleatum*) and nonselective media (Columbia Blood Agar for *Actinomyces oris*, *Veillonella dispar* and total CFU) and colonies were counted.

Distilled water was used as the negative antimicrobial control, and 0.2% chlorhexidine-gluconate solution (Sigma-Aldrich, Steinheim, Germany) in water was the positive antimicrobial control. In order to discard a possible antimicrobial effect of the alcohol, a 12%-ethanol-in-water solution was also tested. For both, test solutions and controls, experiments were carried out in triplicate.

Assay for Assessing Phenolic Metabolism in the Biofilm. After initiating biofilm formation as described above, the 70:30 saliva:mFUM media was enriched with the wine extract (1.6 g/L) in the absence of the presence of grape seed extract (10 g/L) and added into the wells containing the discs (Figure 1). Then, plates were incubated at 37 °C under anaerobic conditions and aliquots of enriched media were taken at 0, 2, 4, 6, 8, and 24 h.

Analysis of Wine Compounds and Bacterial/Microbial Metabolites. Phenolic compounds were analyzed using an UPLC-ESI-MS/MS following a previously reported method.[20] The liquid chromatographic system was a Waters Acquity UPLC (Milford, MA) equipped with a binary pump, an autosampler thermostated at 10 °C, and a heated column compartment (40 °C). The column employed was a BEH-C18, 2.1 × 100 mm and 1.7 μm particle size from Waters (Milford, MA). The mobile phases were 2% acetic acid in water (A) and 2% acetic acid in acetonitrile (B). The gradient program was as follows: 0 min, 0.1% B; 1.5 min, 0.1% B; 11.17 min, 16.3% B; 11.5 min, 18.4% B; 14 min, 18.4% B; 14.1 min, 99.9% B; 15.5 min, 99.9% B; 15.6 min, 0.1% B. Equilibrium time was 2.4 min resulting in a total runtime of 18 min. The flow rate was set constant at 0.5 mL/min and injection volume was 2 μL.

The LC effluent was pumped to an Acquity TQD tandem quadrupole mass spectrometer equipped with a Z-spray electrospray ionization (ESI) source operated in negative polarity mode. The ESI parameters were set as follows: capillary voltage, 3 kV; source temperature, 130 °C; desolvation temperature, 400 °C; desolvation gas (N_2) flow rate, 750 L/h; cone gas (N_2) flow rate, 60 L/h. The ESI was operated in negative ionization mode. For quantification purposes, data were collected in the multiple reaction monitoring (MRM) mode, tracking the transition of parent and product ions specific to each compound. The MS/MS parameters (cone voltage, collision energy and MRM transition) of the 60 phenolic compounds targeted in the present study (mandelic acids, benzoic acids, phenols, hippuric acids, phenylacetic acids, phenylpropionic acids, cinnamic acids, 4-hydrox-

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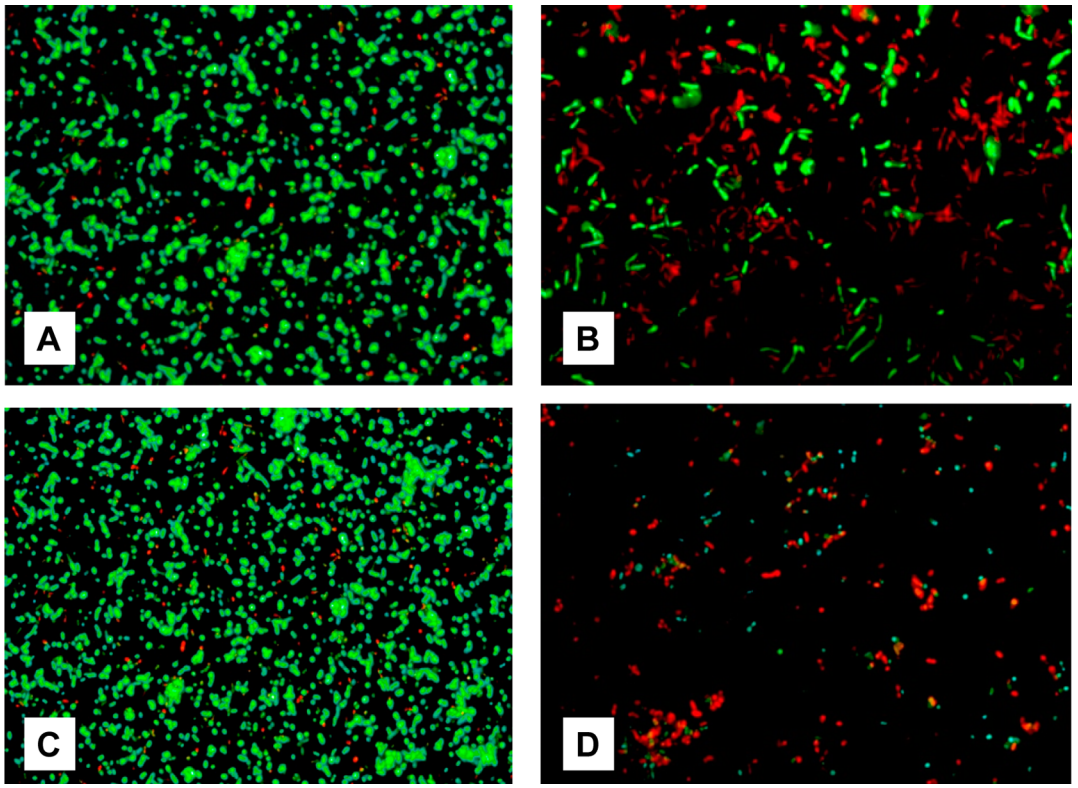

Figure 2. Confocal scanned segment of biofilm stained with LIVE/DEAD kit after exposure to (A) negative control (water), (B) red wine, (C) wine extract (Provinols, 1.6 g/L), and (D) grape seed extract (Vitaflavan, 2.5 g/L) in wine extract solution (1.6 g/L).

Table 2. Bacterial Populations (Log_{10}CFU) of *S. mutans*, *S. oralis*, *A.oris*, *F. nucleatum*, and *V. dispar* in the Biofilm, after Treatments with Water, Ethanol 12% in Water, Wine, Dealcoholized Wine and 0.2% Clorhexidine-gluconate

	S. mutans	*S. oralis*	*F. nucleatum*	*A. oris*	*V. dispar*
Water	8.09 ± 0.09	8.42 ± 0.17	5.90 ± 0.89	8.40 ± 0.32	7.36 ± 0.37
Ethanol 12% in water	8.01 ± 0.16	8.20 ± 0.37	<1.30 ± 0.00[a]	8.75 ± 0.64	7.92 ± 0.12
Wine	7.89 ± 0.07	5.77 ± 0.63[a]	<1.30 ± 0.00[a]	8.37 ± 0.20	6.94 ± 0.38
Dealcoholized wine	7.68 ± 0.22	4.79 ± 0.80[a]	<1.30 ± 0.00[a]	8.24 ± 0.07	7.12 ± 0.88
Clorhexidine-gluconate 0.2%	<1.30 ± 0.00[a]	<1.30 ± 0.00[a]	<1.30 ± 0.00[a]	<1.30 ± 0.00[a]	<1.30 ± 0.00[a]

[a]Significant differences (Dunnett's test) in the population in comparison to the negative control (water).

yvaleric acids and valerolactones) were previously reported.[24] Data acquisition and processing was realized with MassLynx 4.1 software.

Staining of Biofilms and Confocal Laser Scanning Microscopy (CLSM). For CLSM, treated as well as untreated, biofilms were stained using the LIVE/DEAD BacLight bacterial viability assay (Invitrogen, Zug, Switzerland) according to the instructions of the manufacturer. After 20 min staining, excess dye was gently aspirated from the discs without touching the biofilms. They were embedded upside-down in 20 μL of Mowiol[25] and stored at room temperature in the dark for at least 6 h prior to microscopic examination.

Stained biofilms were examined by CLSM at randomly selected positions using a Leica TCS SP5 (Leica Microsystems, Heidelberg GmbH, Germany) with a ×20/0.8 numerical aperture (NA) and a ×63/1.4 NA oil immersion objective lens in conjunction with 488 nm laser excitation and 530 nm emission filters for Syto 9 (live stain) and 561 nm laser excitation, and 640 nm emission filters for propidium iodide (dead stain). Image acquisition was done in 8-line average mode, and the data were processed using Imaris 7.2.2 (Bitplane AG, Zurich, Switzerland).

Statistical Analysis. Means and standard deviations were calculated using Microsoft Excel 2007. Statistical analyses were performed through Statistica©. To compare the antimicrobial effects of the different treatments with the control (water), the Dunnett test was applied. Graphs were performed with Microsoft Excel 2007.

RESULTS AND DISCUSSION

Antimicrobial Properties of Red Wine on the Biofilm. The effects of a red wine and the same wine without ethanol on a biofilm model composed of five representative species commonly encountered in supragingival plaque, including Gram-positive (*A. oris, S. mutans, S. oralis*) as well as Gram-negative (*F. nucleatum, V. dispar*) bacteria,[21] were investigated. Among these bacteria were the so-called early colonizers, *A. oris*, *S. oralis* and *V. dispar*, and late colonizers, *S. mutans* and *F. nucleatum*, the latter also designated as a bridging organism due to its capability to coaggregate with a wide range of early and late colonizers.[26] In comparison to the control biofilm (Figure 2A), when biofilms were dipped into red wine (Figure 2B) and dealcoholized red wine, some decrease in cell viability of the whole biofilm was visually estimated. However, no visually changes were observed when applying the wine extract solution (Figure 2C). CFU values for the five bacteria comprising the biofilm indicated an important reduction in *F. nucleatum* and *S. oralis* population when applying red wine and dealcoholized red wine to the biofilm, in comparison to the negative control (distilled water) (Table 2). The Dunnett test confirmed

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Table 3. Bacterial Populations ($Log_{10}CFU$) of *S. mutans*, *S. oralis*, *A.oris*, *F. nucleatum*, and *V. dispar* in the Biofilm, after Treatments with Provinols, Provinols + Vitaflavan, Provinols + IDY 1 and Provinols + IDY 2

	S. mutans	*S. oralis*	*F. nucleatum*	*A. oris*	*V. dispar*
Water + 2.5% DMSO	8.02 ± 0.05	8.47 ± 0.09	6.77 ± 0.07	8.55 ± 0.07	7.74 ± 0.04
Provinols	8.11 ± 0.08	8.59 ± 0.11	6.54 ± 0.57	8.34 ± 0.39	7.68 ± 0.39
Provinols + Vitaflavan	7.77 ± 0.18	6.49 ± 0.07[a]	<1.30 ± 0.00[a]	<3.30 ± 0.00[a]	7.95 ± 0.09
Provinol + IDY 1	8.18 ± 0.03	8.60 ± 0.01	7.13 ± 0.13	8.89 ± 0.02	8.15 ± 0.15
Provinols + IDY 2	8.13 ± 0.07	8.44 ± 0.07	7.14 ± 0.04	8.68 ± 0.03	8.11 ± 0.11

[a]Significant differences (Dunnetts test) in the population in comparison to the negative control (water + 2.5% DMSO).

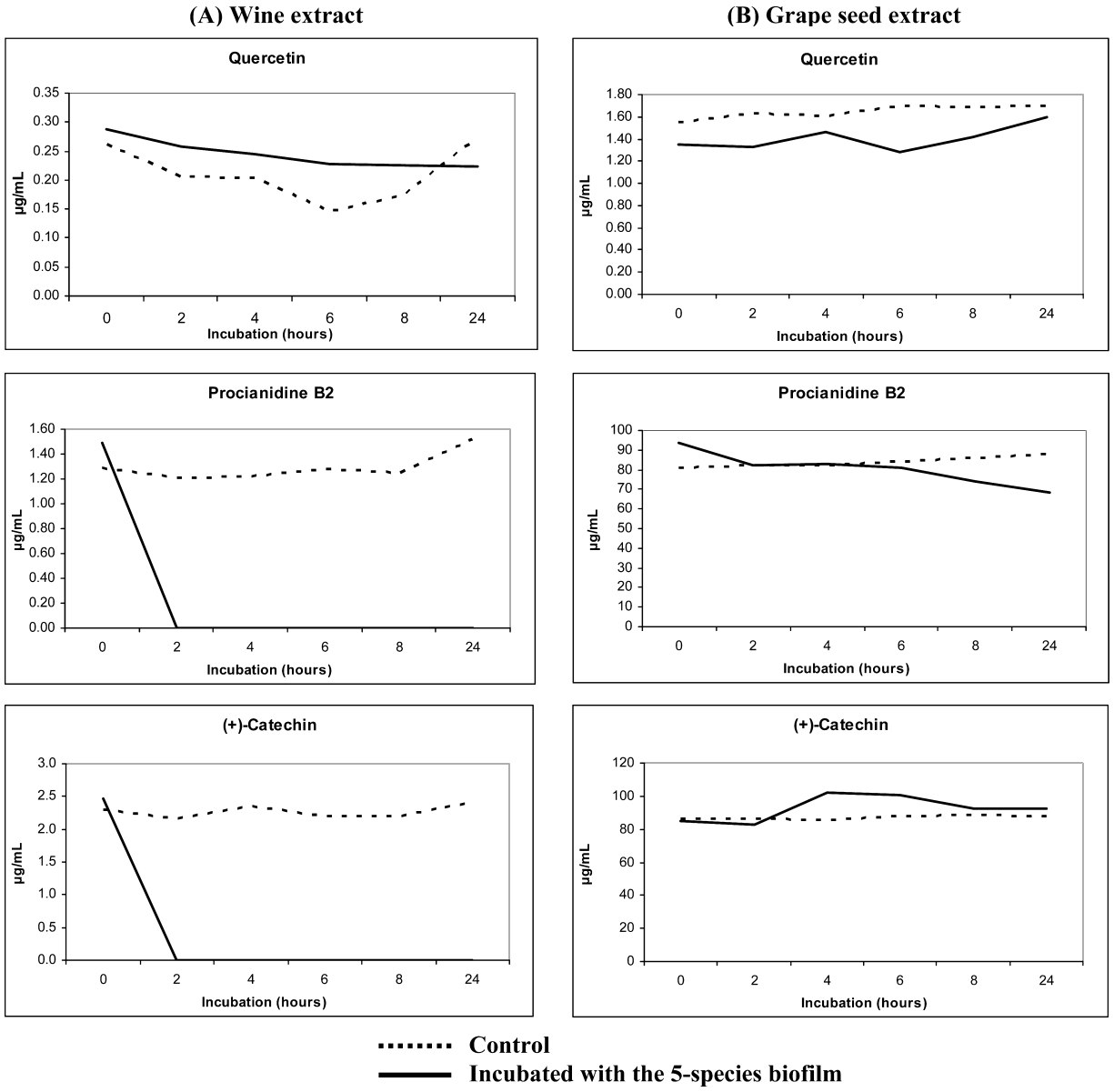

Figure 3. Metabolism of precursors (+)-catechin, quercetin and procyanidin B2 after 0, 2, 4, 6, 8, and 24 h of incubation in FUM media enriched with (A) wine extract (Provinols) and (B) grape seed extract (Vitaflavan, 1%) in wine extract solution.

significant differences in the population of these two strains after the treatment with wine and dealcoholized wine. Generally, wines contain between 10 and 12% of ethanol, which have antimicrobial properties. To understand the action mechanism of red wines in more depth, the effects of ethanol of the bacteria biofilm were investigated. The treatment with 12% ethanol resulted in a significant decrease in the population of *F. nucleatum* (Table 2). However, since treatments of the biofilm with both wine and dealcoholized wine inhibited *F. nucleatum* growth, it was likely that other wine components—apart from ethanol—had antimicrobial properties against this bacteria species. As expected, all the strains were eradicated after the treatment with the positive control (0.2% chlorexidine-gluconate solution) (Table 2).

In an intervention study with 75 volunteers, Signoretto et al.[27] analyzed the microbial population of supragingival and

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subgingival plaque using PCR-DGGE and found that *F. nucleatum* was less frequent in wine drinkers compared with water drinkers. Other authors, such as Daglia et al.,[28] have also shown antimicrobial properties of dealcoholized wine against oral streptococci. Both studies were consistent with our results in that wine selectively inhibited the growth of *F. nucleatum* and *S. oralis* in the presence of other species, such as *S. mutans*, *A. oris* and *V. dispar* in an oral biofilm model.

Given the antimicrobial effects of wine observed in the first experiment, the next step was to study the influence of some wine-specific components, such as polyphenols, including flavan-3-ols, peptides or yeast polysaccharides. For that purpose, a red wine extract solution spiked with different extracts rich in those specific components of wine (grape seed extract rich in flavan-3-ols, and two inactive dry yeasts rich in peptides and mannoproteins, respectively) was used. Table 3 reports the CFU values of the five bacteria species of the tested biofilm after treatments with wine extract and wine extract solution spiked with different extracts (grape seed extract, IDY1 and IDY2). Dunnett's test showed significant differences in *F. nucleatum, S. oralis and A. oris* with the application of the wine extract spiked with the grape seed extract rich in flavan-3-ols.[17] However, wine extract solutions spiked with IDY1 and IDY2 did not show any effect in the populations of the five-strain biofilm. Notably, a great decrease in the viability of the cell was visually appreciated in the biofilm recovered from the discs that were dipped in the grape seed extract solution (Figure 2D). Cueva et al.[29] reported significant inhibition in the growth of some oral streptococci, such as *Streptococcus mutans* and *Streptococcus sobrinus*, when incubating planktonic cultures with flavan-3-ols precursors, (+)-catechin and (−)-epicatechin, in which grape seed extract is particularly rich. Moreover, they showed that extracts from grape seed, especially Vitaflavan and its oligomeric fraction, exerted higher antimicrobial activity against various oral pathogens than the rest of the extracts tested (red wine extract and grape pomace extract). Similarly, Rotava et al.[30] and Baydar et al.[31] reported antimicrobial effects of grape seed extracts against pathogenic bacteria such as *S. aureus* and *E. coli*. It has been suggested that the high concentration of flavonoids and their derivatives in grape seeds could be responsible for the antimicrobial activity of grape seed extracts.[32] These observations raise the question of how the hydroxyl groups (structure) of flavonoids affect oral bacterial biofilm.

The search for new antimicrobial agents to control the formation of dental plaque requires appropriate screening models that include orally relevant organisms. The mode used in this study is not only useful for investigating ecological shifts in plaque composition in response to plaque composition but also for testing the efficacy of antimicrobial agents under conditions of repeated short-term exposure.[11] Other oral biofilm models that use flow chamber systems or in-mouth dispositives have also been used. Among these, flow chamber systems allowing biofilm formation/maturation under hydrodynamic conditions, and careful control and easy changes of the environment, are very useful for analysis of structural biofilm formation. In-mouth dispositives allow *in vivo* formation of the biofilm including the endogenous microbiota of the volunteer. Although these models are the most realistic in miming oral biofilms, the high interindividual variability in the composition of the biofilms may provoke high deviations in the results.

Changes in Wine Phenolic Metabolism. Because wine and its polyphenols diminished bacteria population in the oral biofilm, a new assay was performed in order to gain a deeper understanding about microbial metabolism of polyphenols in the tested extracts.

First, the wine extract solution was added to the growth media and the progress of the phenolic metabolism by the five-species biofilm was studied by monitoring changes in the main phenolic compounds present in the wine extract (Table 1): flavan-3-ols monomers ((+)-catechin, (−)-epicatechin and (−)-epicatechin-3-*O*-gallate), dimeric procyanidins (B1, B2, B3, B4, B5, B7, B2-3-*O*-gallate and B2-3-*O*-gallate), trimeric procyanidins (C1 and other trimers) and flavonols (quercetin, myricetin, kaempherol, quercetin-3-*O*-glucoside and quercetin-3-*O*-galactoside). As a brief example, Figure 3A shows the differences in the degradation by the five-strain biofilm of three of the analyzed precursors, (+)-catechin, procyanidin B2 and quercetin, when growing in media enriched with the wine extract solution (1.6 g/L). The UPLC-MS analysis of these three compounds showed high degradation rates, almost completely during the first 2 h of incubation, in the flavan-3-ol precursors, (+)-catechin and procyanidin B2, probably because of their low concentration in the media, which permitted the bacteria of the biofilm to ues them as a carbon source. However, no degradation of the precursor quercetin was observed during the incubation period.

To gain further knowledge about the metabolism of grape polyphenols, specifically in flavan-3-ols metabolism, the growing media was enriched by adding, to the red wine extract solution, a concentration of 10 g/L of grape seed extract, which is especially rich in flavan-3-ol precursors (Figure 3B). Despite the greater concentration of flavan-3-ol precursors, no degradation of the flavan-3-ol precursors was observed. Nor was degradation of precursor quercetin observed during the incubation period.

The emergence of antibiotic resistance by some oral bacteria biofilm species presents a worldwide problem, and thus, novel strategies are required. The use of natural antimicrobials may contribute to controlling the disordered growth of oral microbiota; thus, overcoming problems caused by species resistant to conventional antimicrobials.[33] To our knowledge, this is the first report on the antimicrobial properties of wine in an oral biofilm model. Our results show that red wine, at moderate concentration, inhibits the growth of some pathogenic species in an oral biofilm model. These findings contribute to existing knowledge about the beneficial effects of red wines (one of the most important products of agriculture and food industries) on human health. Moreover, the promising results concerning grape seed extract, which showed the highest antimicrobial activity, open promising ways toward a natural ingredient in the formulation of oral care products specifically indicated for the prevention of caries, due to its antimicrobial properties.

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