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Antibacterial Activity of Red and White Wine against Oral Streptococci

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Wine contains a number of biologically active compounds with beneficial effects on human health. The antibacterial action of commercial red and white wines against oral streptococci responsible for caries development and against *S. pyogenes* responsible for pharyngitis was studied. Its postcontact effect against *S. mutans* was also studied. Both wines displayed activity. The compounds responsible for such activities were succinic, malic, lactic, tartaric, citric, and acetic acid. The synthetic mixtures of the organic acids tested at the concentrations found in wine had greater antibacterial activity than the beverages, indicating that in wine they are inhibited by other components. Wine polyphenols displayed no activity against oral streptococci or *S. pyogenes*. Findings show that wine is active against oral streptococci and *S. pyogenes* and suggest that it enhances oral health.

KEYWORDS: Red and white wine; oral streptococci; antibacterial activity; organic acids

INTRODUCTION

Several studies suggest that moderate wine consumption has beneficial effects on human health. The antioxidant and antiradical properties, particularly of red wine, attributed mainly to a high polyphenol content (*1*−*3*), appear to protect against the risk of coronary heart disease and cancer.

Wine also possesses antimicrobial properties. Weisse et al. (*4*) reported that red and white wines are as potent as bismuth salicylate against several bacteria responsible for traveler's diarrhea and that diluted ethanol induced no significant reduction in colony counts. Sugita-Konishi et al. (*5*) showed the in vitro antibacterial activity of red and white wines against three potential entero-pathogenic bacteria; the activity was exerted by polyphenol free fractions and was lost after the evaporation of small molecules, particularly acetic acid, suggesting that this small molecule could be responsible for the antibacterial activity. Similarly, Dolara et al. (*6*) found antibacterial activity against selected Gram-positive and Gram-negative pathogenic bacteria by two industrial and a homemade wine (produced by spontaneous fermentation with no added synthetic chemicals). The greater activity of the two industrial wines suggested that this action is not accounted for by bisulfite addition in the industrial process; moreover, the effect was not caused by polyphenols, ethanol, or the acidic pH induced by wine in culture media, whereas acetic acid, a common wine constituent, was seen to induce an inhibitory effect similar to that of wine. In an investigation of the antimicrobial activity of 16 Chilean red wines against 6 strains of *Helicobacter pylori* from gastric biopsies, the main active compound was found to be resveratrol, a stilbene derivative that in plants acts as a phytoalexin (*7*). Resveratrol was also active against bacteria and dermatophytes, which are major etiologic agents in human skin infections (*8*). Navarro et al. (*9*) showed that several lactic acid bacteria occurring in Rioja red wine produced bacteriocins, antimicrobial peptides, during alcoholic and malolactic fermentation of wine.

Several strains of oral streptococci are capable of initiating the formation of dental plaque, which plays an important role in the development of caries and periodontal disease in humans. *Streptococcus mutans*, a potent cariogenic, can colonize tooth surfaces and initiate plaque formation by its ability to synthesize extracellular polysaccharides from sucrose, mainly water-insoluble glucan, using glucosyltransferase (*10*). Streptococcal growth inhibition by antibacterial agents has extensively been investigated. The antibiotic and chemical bactericides currently used to prevent bacterial infection often disturb the bacterial flora of the oral cavity and digestive tract (*11*). A small number of recent studies have reported antimicrobial activity of natural agents against selected oral pathogens. Ceanothic acid and ceanothetric acid from the native American plant *Ceanothus americanus* has been shown to inhibit the growth of *S. mutans*, *Actinomyces viscosus*, and *Porphyromonas gingivalis* (*12*). Propolis ethanolic extract exerts in vitro antibacterial action against a number of oral microorganisms and inhibits cell adhesion as well as water-insoluble glucan formation (*13*). Bakuchiol, a phenolic isoprenoid derived from the seeds and leaves of the tree *Psoralea corylifolia* L., native to China, showed a bactericidal effect against oral bacteria (*14*). Extracts obtained from different teas affect caries development, as their

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polyphenol components reduce the production of acidic compounds and the ability of streptococci to synthesize adherent water-insoluble glucan from sucrose with the cooperative action of glucosyltransferase (*15*−*17*). In vivo rat studies of the extract from *Lentinus edodes*, the most popular edible mushroom in Japan (*18*), showed an inhibitory effect on water-insoluble glucan formation by glucosyltransferase. A similar effect has been described for apple procyanidins in vitro (*19*) and for hop bract high molecular weight components (*20*). Cacao mass extract displayed cariostatic activity in vitro and in animal experiments (*21*). Onion has bactericidal activity against *Porphyromonas gingivalis* and *Prevotella intermedia* (*22*). Research by our group evidenced an antibacterial activity of coffee against Gram-positive and -negative bacteria (*23*−*25*) that seems to be due to a small molecule induced by roasting of the beans. In particular, roasted coffee interferes with streptococcal sucrose-independent adsorption to hydroxyapatite (HA) beads, also through the involvement of small molecules, such as trigonelline and nicotinic and chlorogenic acids (*26*).

Naturally effective antimicrobial agents against oral pathogens could play an important role in preventing dental caries. The activity of wine against oral bacteria has not yet been investigated. The present study was undertaken to explore the antibacterial activity of red and white commercial wines against several strains of oral streptococci responsible for dental plaque formation and caries development and against *S. pyogenes*, which causes a wide range of human diseases, such as pharyngitis. After establishment of the bacteriostatic activities of the two wines, their active components were investigated.

MATERIALS AND METHODS

Chemicals. Potassium phosphate buffer (PB), sodium hydroxide, ethyl acetate, methanol, diethyl ether, 5-*O*-caffeoylquinic acid (5-*O*-CQA), tartaric acid, malic acid, citric acid, lactic acid, acetic acid, succinic acid, and hydrochloric acid were purchased from Sigma-Aldrich (Milan, Italy).

Wine Samples. An Italian red wine, Valpolicella Classico DOC Superiore, 2003 vintage (pH 3.56, 13.5% alcohol), and an Italian white wine, Pinot Nero DOC 2003 vintage (pH 3.82, 11.5% alcohol), were purchased from a local supermarket.

Bacterial Strains, Media, and Buffers. The following streptococcal strains were used: *S. mutans* 9102 (*26*), *S. vestibularis* ATCC 49124, *S. anginosus* ATCC 33397, *S. intermedius* ATCC 27335, *S. constellatus* ATCC 27823, *S. oralis* ATCC 10557, *S. salivarius* ATCC 13419, *S. sanguinis* ATCC 10556, and *S. pyogenes* ATCC 19615. Bacteria were cultured in Todd Hewitt broth (THB, Oxoid, Basingstoke, U.K.) at 37 °C in the presence of 5% CO_2.

Evaluation of Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC). Following dealcoholization by vacuum concentration (50 mbar) at 40 °C, the dealcoholized wines (red, RDW; white, WDW) were filtered through a Millex GP membrane (0.22 μm) (Millipore Corp., Billerica, MA). MICs and MBCs were determined in Iso-Sensitest broth (ISB, Oxoid) according to Clinical and Laboratory Standards Institute (CLSI; formerly NCCLS) procedures (*27*). The MIC was the lowest dealcoholized wine (DW) concentration inhibiting observable growth; the MBC was the lowest concentration resulting in >99.9% reduction of the initial inoculum (*28*). All experiments were performed in triplicate.

Evaluation of Postcontact Effect (PCE). Aliquots of bacterial cultures grown in ISB to the exponential phase (approximately 10^8 CFU mL^{-1}) were exposed to 1 × and 2 × MIC of DWs or to the organic acid aqueous solutions for 1 h at 37 °C; control cultures were left untreated. Exposed and control cultures were washed twice in PB by centrifugation at 3500 rpm for 20 min and then diluted in fresh broth before incubation at 37 °C. Viable counts were measured in tryptone soy agar (TSA; Oxoid) immediately and 1, 2, 3, 4, 5, and 24 h after incubation. PCE was determined using the equation

$$\mathrm{PCE} = t - c \quad (1)$$

where t was the time needed for a log increase in counts in treated cultures and c the time needed for a log increase in counts in untreated cultures (*29*). The samples were tested on *S. mutans*. All experiments were performed in triplicate.

Solid-Phase Extraction (SPE). SPE was performed according to the method of Sun et al. (*30*), with some modifications. A 5 mL aliquot of RDW or dialysate was concentrated to dryness in a rotary evaporator at <30 °C. The residue was dissolved in 20 mL of PB (pH 7.0) and adjusted to pH 7.0 with NaOH solution. Two C_{18} 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 of PB, pH 7.0 (SPE-F1 or D-SPE-F1). After the cartridges were dried with N_2, simultaneous elution of monomeric and oligomeric flavan-3-ols (SPE-F2 or D-SPE-F2 and SPE-F3 or D-SPE-F3) was obtained with 25 mL of ethyl acetate followed by elution of polymeric proanthocyanidins (SPE-F4 or D-SPE-F4) with 15 mL of methanol. The ethyl acetate fraction was taken to dryness under vacuum, redissolved in 3 mL of PB, and finally redeposited onto the same conditioned cartridges. Cartridges were dried with N_2, and monomers (SPE-F2 or D-SPE-F2) were separated from oligomers (SPE-F3 or D-SPE-F3) by sequential elution with 25 mL of diethyl ether and 15 mL of methanol. Sample fractionation was performed in duplicate. The four SPE fractions were concentrated to dryness and the residues dissolved in 5 mL of Millipore grade water and then tested on *S. mutans* and *S. pyogenes*. All experiments were performed in triplicate.

Dialysis. Dialysis was performed using a Spectra/Por Biotech cellulose ester membrane (Spectrum Europe B.V., Breda, The Netherlands) with a molecular mass cutoff of 1000 Da. Aliquots (10 mL) of RDW and SPE-F1 were fractionated by dialysis in 1000 mL of Millipore grade water for 6 h at 4 °C. Dialysates and retentates were freeze-dried, and the residues were dissolved in 10 mL of Millipore grade water. Recovered 5-*O*-CQA (>95%) was used as a standard molecular mass marker. Dialysates and retentates were tested against *S. mutans* and *S. pyogenes*.

Organic Acid and Organic Acid Mixture Preparations. Aqueous solutions of acetic, citric, lactic, malic, succinic, and tartaric acid were prepared at the highest concentrations commonly found in wine (*31*): acetic acid, 1.04 mg mL^{-1}; citric acid, 0.64 mg mL^{-1}; lactic acid, 3.86 mg mL^{-1}; malic acid, 2.52 mg mL^{-1}; succinic acid, 3.26 mg mL^{-1}; tartaric acid, 4.03 mg mL^{-1}. Two mixtures containing all of the organic acids but citric acid (because it is produced during alcoholic fermentation in very low concentrations and is not added during winemaking according to the two producers) were prepared: one with the concentrations listed above (HCA mix, pH 2.30 ± 0.03) and the other (LCA mix) with the lowest concentrations commonly found in wine (*32*): acetic acid, 0.19 mg mL^{-1}; lactic acid, 1.93 mg mL^{-1}; malic acid, 0.58 mg mL^{-1}; succinic acid, 0.75 mg mL^{-1}; tartaric acid, 1.34 mg mL^{-1} (pH 2.75 ± 0.04). The solutions were analyzed for antibacterial (MIC) and bactericidal (MBC) activity against *S. mutans* and *S. pyogenes* and for PCE against *S. mutans*.

Statistical Analysis. The values represent a mean value of at least three replications. Data were analyzed using the analysis of variance test (ANOVA) with the statistical package Statgraphics Plus (1998). Means were separated with the LSD method at a confidence level of 95%.

RESULTS AND DISCUSSION

Antibacterial Activity of Wine toward Oral Streptococci and *S. pyogenes*. Two commercial Italian wines, a red wine (Valpolicella Classico DOC Superiore) and a white wine (Pinot Nero DOC), were tested for antibacterial activity against eight oral streptococci and *S. pyogenes*. They were dealcoholized before microbiological testing to exclude ethanol interference on streptococcal growth. The MICs and MBCs obtained from the dealcoholized beverages, reported in **Table 1**, show that

Table 1. Antibacterial (MIC) and Bactericidal (MBC) Activities of Red and White Dealcoholized Wines against Oral Streptococci and *S. pyogenes*[a]

	red wine		white wine	
strain	MIC (v/v %)	MBC (v/v %)	MIC (v/v %)	MBC (v/v %)
S. anginosus ATCC 33397	20 ± 4	30 ± 2	20 ± 4	30 ± 4
S. constellatus ATCC 27823	10 ± 2	40 ± 4	30 ± 2	50 ± 8
S. intermedius ATCC 27335	10 ± 2	30 ± 2	30 ± 6	40 ± 4
S. mutans 9102	10 ± 2	20 ± 6	20 ± 2	30 ± 2
S. oralis ATCC 10557	10 ± 2	20 ± 2	20 ± 2	30 ± 2
S. salivarius ATCC 13419	20 ± 4	40 ± 6	20 ± 2	40 ± 2
S. sanguinis ATCC 10556	20 ± 2	30 ± 2	20 ± 2	30 ± 4
S. vestibularis ATCC 49124	20 ± 6	40 ± 4	20 ± 2	40 ± 6
S. pyogenes ATCC 19615	10 ± 2	20 ± 2	20 ± 2	30 ± 2

[a] All experiments were performed in triplicate.

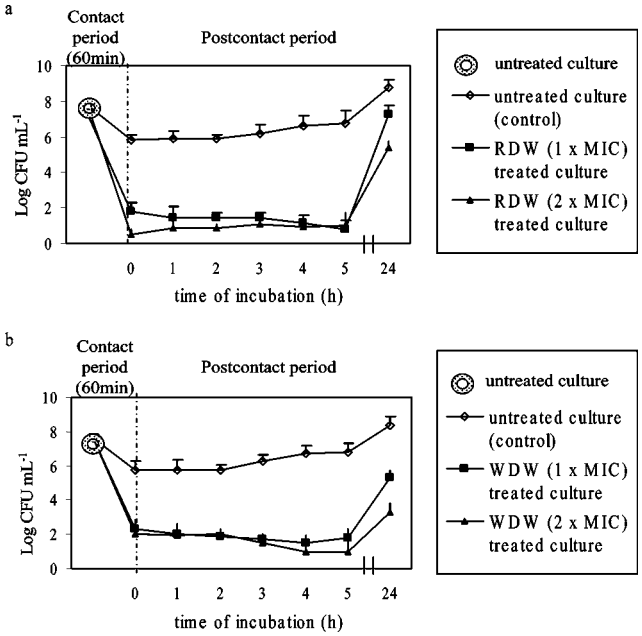

Figure 1. Postcontact effect (PCE) of red (**a**) and white (**b**) dealcoholized wines on *S. mutans* growth.

both wines are active and that the different antibacterial activities are strain dependent. Moreover, RDW has stronger activity than white wine, even if the difference is not statistically significative ($p > 0.05$), as reflected in lower MICs and MBCs (10% < MIC < 20%; 20% < MBC < 40% vs 20% < MIC < 30%; 30% < MBC < 50% of WDW).

To exclude an inhibitory effect due to the acidic pH induced by the dealcoholized beverages in the culture medium (pH 5.00 after addition of 40% v/v DW), the culture medium was acidified to pH 5.00 with 2 N HCl. The lack of growth inhibition observed for all strains confirmed that the antibacterial effect of DWs is due to wine components rather than to the acidic pH induced by it.

PCE of RDW and WDW. To assess whether the effect of DWs is persistent, we investigated PCE on *S. mutans*. Bacteria were exposed to DWs for 60 min, washed, and assessed for PCE on growth. Viable counts decreased after exposure to both DW concentrations of 1 × MIC and 2 × MIC and did not increase for at least 5 h thereafter (**Figure 1**). PCE values were 4.5 and 5.0 at 1 × MIC for RDW and WDW, respectively, and 5.5 and 12.0 at 2 × MIC, respectively, showing a sensitive slowing in growth. Bacterial growth increased after 5 h, but the CFU mL^{-1} values of pretreated cultures at 24 h were lower than in control cultures. In particular, for RDW (2 × MIC) the difference was about 4 log units and for WDW (2 × MIC) it was about 5 log units. These data confirm the bactericidal activity of DW and show a severe effect on the growth rate of surviving bacteria, which had not recovered 24 h from the end of exposure.

Table 2. Antibacterial (MIC) and Bactericidal (MBC) Activities of Red Dealcoholized Wine SPE Fractions (SPE-F) on *S. mutans* and *S. pyogenes*[a]

	S. mutans 9102		*S. pyogenes* ATCC 19615	
SPE fraction RDW	MIC (v/v %)	MBC (v/v %)	MIC (v/v %)	MBC (v/v %)
SPE-F1	10 ± 2	20 ± 4	10 ± 2	20 ± 2
SPE-F2	>50	>50	>50	>50
SPE-F3				
SPE-F4				

[a] All experiments were performed in triplicate.

Table 3. Antibacterial (MIC) and Bactericidal (MBC) Activities of Red Dealcoholized Wine Dialysis Fractions on *S. mutans* and *S. pyogenes*[a]

	dialysate		retentate	
strain	MIC (v/v %)	MBC (v/v %)	MIC (v/v %)	MBC (v/v %)
S. mutans 9102	20 ± 2	30 ± 2	>50	>50
S. pyogenes ATCC 19615	20 ± 2	30 ± 2	>50	>50

[a] All experiments were performed in triplicate.

Isolation and Identification of RDW Antibacterial Compounds. We then turned to the compound(s) responsible for the antibacterial activity detected in wine. For these investigations we used RDW, because of its greater activity compared with WDW.

SPE permits the separation of wine polyphenols from organic acids, residual sugars, and other compounds insoluble in the organic solvent. RDW (5 mL) was concentrated to dryness, and the residue, dissolved in PB, was passed through two preconditioned SPE cartridges connected in series. The most polar compounds were recovered with PB (SPE-F1), then cathechins (SPE-F2) and oligomeric proanthocyanidins (SPE-F3) were eluted simultaneously with ethyl acetate, and finally proanthocyanidins (SPE-F4) were isolated with methanol. Separation of cathechins (SPE-F2) from proanthocyanidins (SPE-F3) was obtained by applying another SPE and sequential elution with diethyl ether (SPE-F2) and then with methanol (SPE-F3). Each SPE fraction (SPE-F1−4), restored to the initial volume of 5 mL, was tested for antibacterial property on *S. mutans* and *S. pyogenes* (**Table 2**). The first fraction (pH 3.50 ± 0.20), containing the most polar compounds eluted with buffer solution (SPE-F1), displayed activity against both streptococci as well as the same MICs and MBCs as the whole RDW; in contrast, all of the other fractions, which contain polyphenol compounds such as cathechin, oligomeric proanthocyanidin, and tannins, showed no activity.

To obtain preliminary indications about the molecular mass (MM) of the compounds responsible for antibacterial activity, RDW was dialyzed using a dialysis membrane with a 1000 Da cutoff, which allowed the separation of low MM wine components (organic acids, monomeric and oligomeric flavan-3-ols, i.e., proanthocyanidin, and low degree of polymerization condensed tannins) from polymeric components, such as high degree of polymerization tannins found in the solid part of the

Table 4. Antibacterial (MIC) and Bactericidal (MBC) Activities of Wine Organic Acids Alone and Mixed[a]

	S. mutans 9102				*S. pyogenes* ATCC 19615			
	MIC		MBC		MIC		MBC	
organic acid	v/v %	μg/mL	v/v %	μg/mL	v/v %	μg/mL	v/v %	μg/mL
acetic acid	21.0 ± 2	220.2	70.0 ± 8	419.5	40.0 ± 2	734.2	80.0 ± 6	839.0
citric acid	20.0 ± 2	128.0	60.0 ± 6	256.0	40.0 ± 4	384.0	80.0 ± 6	512.0
lactic acid	3.0 ± 1	115.8	20.0 ± 2	231.6	6.0 ± 1	772	30.0 ± 2	1158.0
malic acid	4.0 ± 1	100.8	30.0 ± 2	151.2	6.0 ± 2	756.0	40.0 ± 2	1008.0
succinic acid	3.0 ± 2	97.8	20.0 ± 1	163.0	5.0 ± 1	652.0	30.0 ± 2	978.0
tartaric acid	3.0 ± 2	120.9	20.0 ± 2	241.8	6.0 ± 2	806.0	30.0 ± 2	1209.0
HCA mix[b]	0.8 ± 0.2		5.0 ± 1		2.0 ± 1		9.0 ± 1	
LCA mix[c]	7.0 ± 2		20.0 ± 2		10.0 ± 2		20.0 ± 2	

[a] All experiments were performed in triplicate. [b] HCA mix: organic acid mixture prepared with the highest concentrations commonly found in red wine; without citric acid. [c] LCA mix: organic acid mixture prepared with the lowest concentrations commonly found in red wine; without citric acid.

grape (skin, seeds, and stems) and extracted during winemaking. Dialysates and retentates were tested after restoration of the initial wine volume, so that their constituents were tested at the same concentrations at which they are found in the beverage.

Results (**Table 3**) showed that only the dialysate ($pH_{dialysate}$ 3.50 ± 0.20), containing low MM components, possesses any antibacterial activity and that it was slightly weaker than the wine's, as demonstrated by its higher MICs and MBCs. These data confirm that condensed high degree of polymerization tannins exert no antibacterial activity against oral streptococci and suggest that some antibacterial agents were lost during the dialysis process or, more likely, during vacuum concentration in the rotary evaporator.

The dialysate (MM < 1000 Da) was further fractionated by the SPE process previously applied to wine. Again, only the first fraction (D-SPE-F1, pH 3.48 ± 0.20) was active and had the same MICs and MBCs as the whole dialysate, indicating that low molecular mass polar and acidic compounds are responsible for the antibacterial activity of wine.

Antibacterial Activity of Wine Components toward Oral Streptococci and *S. pyogenes*. We thus turned to study acetic, citric, lactic, malic, succinic, and tartaric acid, which are found in grapes (*31*) or are produced during malolactic fermentation (*32*). The MICs and MBCs of the standard aqueous organic acid solutions were evaluated against the test bacteria. MIC and MBC values (**Table 4**) showed that at the concentrations commonly found in red and white wines (*32*, *33*) these acids can be responsible for the activities documented in our experiments. Therefore, the antibacterial activity described in other papers is not exerted by acetic acid alone (*5*, *6*). When we tested the two mixtures of active organic acids at the highest (HCA mix) and lowest (LCA mix) concentrations reported in the literature for red wine (*32*), they displayed much lower MICs and MBCs than DW on both bacteria. The activity of DW is thus to be attributed to its organic acid action even though it seems to be inhibited by other wine components.

The PCE of the organic acids and the HCA mix on *S. mutans* was also evaluated at 1 × MIC and 2 × MIC concentrations (**Table 5**). The standard solutions had different PCEs on growth, with succinic and tartaric acid displaying none, whereas the HCA mix achieved greater PCE than both the organic acid solutions and DWs.

In conclusion, both red and white wines were proved to exert in vitro antibacterial activity against several oral streptococci and *S. pyogenes* and to induce postcontact effects against *S. mutans*. Succinic, malic, lactic, tartaric, citric, and acetic acid all exhibited antibacterial and postcontact activities and can therefore be collectively considered to be responsible for these properties. Conversely, wine polyphenols displayed no activity against the microorganisms.

Table 5. Postcontact Effect on *S. mutans* of Organic Acids Alone and Mixed[a]

	postcontact effect (h)	
organic acid	1 × MIC	2 × MIC
acetic acid	6.0	7.0
citric acid	6.0	11.0
lactic acid	8.0	12.0
malic acid	4.2	4.2
succinic acid	na[b]	0.2
tartaric acid	na	na
HCA mix	11.0	14.0

[a] All experiments were performed in triplicate. [b] No activity,

The MICs and MBCs of the organic acid mixture at the concentrations found in red wine are much lower than those of the beverage, indicating that in wine the activities of the organic acids are partly inhibited.

The PCE experiments showed that exposure to wine had a persistent antibacterial effect at the time points investigated. This could reflect the interval in which cells regenerate active enzyme molecules after dissociation of the bond of DW's bioactive compounds from the target site. Overall, our findings seem to indicate that wine can act as an effective antimicrobial agent against the tested pathogenic oral streptococci and might be active in caries and upper respiratory tract pathologies prevention. Research is in progress to evaluate the effects of wine on oral streptococci in vivo and its influence on *S. mutans* adherence to tooth surfaces and on bioadhesive glucan production.

ABBREVIATIONS USED

DW, dealcoholized wine; RDW, red dealcoholized wine; 5-*O*-CQA, 5-*O*-caffeoylquinic acid; PB, potassium phosphate buffer; MIC, minimal inhibitory concentration; MBC, minimal bactericidal concentration; SPE, solid-phase extraction; SPE-F, solid-phase extraction fraction obtained from red dealcoholized wine; D-SPE-F, solid-phase extraction fraction obtained from red dealcoholized wine dialysate; THB, Todd Hewitt broth; ISB, Iso-Sensitest broth; TSA, tryptone soy agar; PCE, postcontact effect; MM, molecular mass; HCA mix, highest concentration organic acid mixture; LCA mix, lowest concentration organic acid mixture.

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