JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

Cite This: J. Agric. Food Chem. XXXX, XXX, XXX−XXX

pubs.acs.org/JAFC

Inhibition of Oral Pathogens Adhesion to Human Gingival Fibroblasts by Wine Polyphenols Alone and in Combination with an Oral Probiotic

Adelaida Esteban-Fernández,[†] Irene Zorraquín-Peña,[†] Maria D. Ferrer,[‡,§] Alex Mira,[‡,§] Begoña Bartolomé,[†] Dolores González de Llano,[†] and M. Victoria Moreno-Arribas*[,†]

[†]Instituto de Investigación en Ciencias de la Alimentación (CIAL), CSIC-UAM, c/Nicolás Cabrera, 9, 28049 Madrid, Spain

[‡]Department of Health and Genomics, Center for Advanced Research in Public Health, FISABIO Foundation, 46020 Valencia, Spain

[§]CIBER Epidemiology and Public Health, 28029 Madrid, Spain

ABSTRACT: Several benefits have been described for red wine polyphenols and probiotic strains in the promotion of colonic metabolism and health. On the contrary, knowledge about their role in the management of oral health is still scarce. In this work, the antiadhesive capacity of selected red wine polyphenols and oenological extracts against the oral pathogens *Porphyromonas gingivalis*, *Fusobacterium nucleatum*, and *Streptococcus mutans* in an in vitro model of human gingival fibroblasts has been explored as well as their complementary action with the candidate oral probiotic *Streptococcus dentisani*. Results highlighted the antiadhesive capacity of caffeic and *p*-coumaric acids as well as grape seed and red wine oenological extracts. Both, caffeic and *p*-coumaric acids increased their inhibition potential against *S. mutans* adhesion when combined with *S. dentisani*. Additionally, UHPLC−MS/MS analysis demonstrated the oral metabolism of wine phenolics due to both, cellular and bacterial activity.

KEYWORDS: *wine polyphenols, oral bacteria, probiotics, cell adhesion, oral health, metabolism*

INTRODUCTION

Polyphenols are defense secondary metabolites found in numerous plant species and their fruits. Their antioxidant activity has been widely studied; however, other beneficial properties have been described for polyphenol-rich food, including promotion of cardiovascular health, protective effect in neurodegenerative disorders, and metabolic diseases prevention.[1] Red wine is a rich source of dietary polyphenols which possesses a unique combination of phenolic structures (mainly flavonoids but also nonflavonoids).[2] Several intervention studies in humans and animals have provided further evidence of the protective effects of moderate wine consumption (~250 mL per day), on cardiovascular diseases, diabetes, and neurodegenerative disorders as well as in promotion of gut health among others.[2−4] Phenolic components in wine may also have an effect on human microbiota. In particular, different studies have recently shown that red wine consumption can significantly modulate the growth of selected bacteria of colonic microbiota in healthy humans.[5−7]

Oral microbiota is characterized by a high variability and abundance, containing more than 700 species.[8] Most oral bacteria are located in dental plaque, in a biofilm structure attached to hard and soft tissues,[9] due to the production of microbial exopolysaccharides (EPSs). Therefore, attachment to buccal surfaces is a key step on the development of microbial-derived oral pathologies and it takes place in two steps: first, initial surface attachment by primary colonizers (usually streptococci), which originates a microbial monolayer; then, migration of these colonizers and addition of secondary and late colonizers (i.e., *Fusobacterium nucleatum, Porphyromonas. gingivalis*),[10] which leads to the formation of a multilayered matrix. However, the oral cavity is a dynamic open system which can easily change. Environmental stimuli (i.e., host susceptibility, poor oral hygiene or dietary habits) can modify oral microbiota, altering the natural balance between commensal and pathogenic microorganisms. This imbalance could be translated into an overgrowth of pathogenic population on detriment of commensal microbes, resulting in a shift of microbial ecology and subsequent development of microbial origin oral diseases.[10] Among them, caries and periodontal diseases (periodontitis and gingivitis) can be distinguished. Caries disease results on the dissolution of the tooth enamel due to excessive organic acids production (low pH conditions) by pathogenic bacteria, such as *Streptococcus mutans* and *Streptococcus. sobrinus*, considered as starters of the lesion.[11] On the other hand, periodontal diseases are characterized by an increase of Gram-negative species (i.e., *P. gingivalis*, *Campylobacter* spp., *Treponema denticola*, among others) which produce endotoxins which trigger processes of tissue damage, bleeding, inflammation, irritation and, finally, gum detachment.[12] Furthermore, these pathogens are able to display structural moieties that are recognized by host receptors, triggering inflammatory signaling pathways and exacerbated cytokine production.[10] If *S. mutans* plays an essential role as initiator on caries disease, *P. gingivalis* is typically found on chronic periodontitis and has been proposed as the "keystone" microbe in periodontitis initiation, whereas *F. nucleatum* has been mostly described in gingivitis lesions.[10,13]

Traditional therapies used for the maintenance of oral health present some limitations, and the search of natural-origin therapies is gaining attention. In this regard, benefits of polyphenols

Received: November 22, 2017
Revised: January 23, 2018
Accepted: January 25, 2018

DOI: 10.1021/acs.jafc.7b05466
J. Agric. Food Chem. XXXX, XXX, XXX−XXX

as new strategies in the prevention and treatment of microbial derived oral pathologies have been widely described.[10] The main action mechanisms of polyphenols against oral microbial diseases include antiadhesive, antimicrobial, or anti-inflammatory activity as well as inhibition of the expression of proteins responsible of bacterial invasiveness.[10]

The use of probiotics in oral health is limited, and most of them are *bifidobacteria* and lactobacilli, whose natural niche is the gut,[14] which makes it difficult for a successful colonization of the oral cavity. Despite this, the use of probiotics as preventive agents in microbial-derived oral diseases is gaining attention,[15] and some discoveries have pointed out *Streptococcus* strains as potential candidate probiotics in this field.[16] Concretely, *Streptococcus dentisani* 7746 has been isolated from dental plaque of caries-free individuals, and the last evidence[14] has demonstrated that this probiotic expresses bacteriocins against the major oral pathogens, including *S. mutans, Prevotella intermedia*, and *F. nucleatum*. In addition, other beneficial mechanisms of action have been described for this novel isolate, such as buffering ability in acidogenic biofilms in the presence of arginine, a component of some tooth pastes.[14]

Most bacterial phenolic metabolism takes places in the gut, where microbiota transform polyphenols into simple phenols, lactones, and phenolic and aromatic acids,[17] which are believed to be the real executors of the benefits associated with these bioactive molecules. However, this metabolism starts in the oral cavity due to salivary enzymatic action, chewing, and oral microbiota activity.[2] Despite some evidence describing a degradation of red wine and grape seed extracts flavan-3-ols by pathogenic oral biofilm,[18] knowledge about the role of oral components in phenolic metabolism is still preliminary.

In this context, the effect of selected wine phenolic compounds (caffeic and *p*-coumaric acids), and red wine and grape seed extracts (Provinols and Vitaflavan, respectively) on *F. nucleatum, S. mutans*, and *P. gingivalis* adherence to HGF-1 human fibroblasts has been studied. Furthermore, a possible complementary effect between polyphenols and the candidate oral probiotic *S. dentisani* 7746 with anticariogenic ability was explored. Finally, the role of oral bacteria and cells to metabolize polyphenols with the subsequent release of phenolic metabolites has been determined.

MATERIALS AND METHODS

Wine Extracts and Compounds. Two oenological phenolic extracts (Vitaflavan and Provinols) and two pure phenolic metabolites, caffeic acid (Sigma-Aldrich) and *p*-coumaric acid (Extrasynthese, France), were used in this study. Vitaflavan, kindly provided by Dr. Piriou (Dérives Resinique & Terpéniques, S.A., France), is a commercial phenolic extract from grape seeds with a total phenolic content of 629 mg of gallic acid equivalents per g. Provinols, kindly supplied by Safic-Alcan Especialidades S.A.U. (Barcelona, Spain) is a red wine extract with a total phenolic content of 474 mg of gallic acid equivalents per g. The phenolic compositions of both extracts were previously determined by UHPLC–ESI-MS/MS (Table 1)[19,20] for other studies. The same batches of both oenological extracts in the papers referred to in Table 1 were employed. So, the concentrations in Table 1 have been assumed from these analyses and are considered as approximated values.

Bacterial Strains and Culture Conditions. Three bacterial strains including a microaerophilic, *S. mutans* (CECT 479), and two strict anaerobes, *P. gingivalis* (ATCC 33277) and *F. nucleatum* (DSMZ 15643), were selected as cariogenic and periodontal pathogens, respectively. Also, a facultative anaerobe *S. dentisani* 7746 (CECT 8313), previously characterized as oral probiotic,[14] was included.

S. dentisani 7746 and *S. mutans* were cultured in Brain Heart Infusion medium (BHI) (BD, Bergès, France) at 37 °C for 18 h and 5% CO_2 atmosphere in the case of *S. mutans*. Anaerobic bacteria were cultured under anaerobic conditions (90% N_2, 5% CO_2, 5% H_2) at 37 °C in Tryptic Soy Broth medium (TSB) (Scharlau, Barcelona, Spain), supplemented with hemein (5 μg/mL), vitamin K (1 μg/mL), yeast extract (5 mg/mL), and L-cysteine hydrochloride (0.5 mg/mL), according to ATCC specifications. *F. nucleatum* and *P. gingivalis* cultures were grown from fresh inoculum during 18 h and 72 h, respectively.

Table 1. Phenolic Characterization (mg/g) of Provinols (Red Wine Extract) and Vitaflavan (Grape Seed Extract), As Previously Determined by UHPLC–ESI-MS/MS[19,20]

flavan-3-ols and others	Provinols	Vitaflavan[a]
gallic acid	1.06 ± 0.05	9.11 ± 0.01
catechin	9.90 ± 0.32	74.6 ± 0.09
epicatechin	6.87 ± 0.15	67.7 ± 0.75
epicatechin-3-*O*-gallate	0.226 ± 0.018	26.2 ± 0.41
procyanidin B1	11.1 ± 0.1	61.0 ± 1.42
procyanidin B2	4.69 ± 0.10	45.1 ± 0.95
procyanidin B3	1.23 ± 0.02	20.4 ± 0.33
procyanidin B4	0.827 ± 0.018	15.0 ± 0.13
procyanidin B2-3-*O*-gallate	0.0271 ± 0.0106	1.80 ± 0.06
procyanidin B2-3′-*O*-gallate	0.0258 ± 0.0028	1.61 ± 0.01
procyanidin C1	1.07 ± 0.04	7.07 ± 0.08
procyanidin T2	1.24 ± 0.09	6.81 ± 0.06
tyrosol	18.9 ± 1.3	nd
p-coumaric acid[b]	0.36 ± 0.01	0.71 ± 0.18
coutaric acid	2.00 ± 0.12	nd
caftaric acid	0.192 ± 0.071	nd
Anthocyanins		
delphinidin-3-*O*-glucoside	0.568 ± 0.012	nd
cyanidin-3-*O*-glucoside	0.265 ± 0.010	nd
petunidin-3-*O*-glucoside	1.47 ± 0.03	nd
malvidin-3-*O*-glucoside	9.01 ± 0.50	nd
malvidin-3-*O*-(6″-acetyl) glucoside	1.92 ± 0.02	nd
malvidin-3-*O*-(6″-*p*-coumaroyl) glucoside	1.24 ± 0.01	nd
quercetin	22.4 ± 0.6	nd
kaempferol	0.0366 ± 0.0055	nd
myricetin	2.55 ± 0.07	nd
quercetin-3-*O*-glucoside	0.137 ± 0.023	nd
quercetin-3-*O*-galactoside	0.107 ± 0.006	nd
resveratrol	0.427 ± 0.020	nd
resveratrol-3-*O*-glucoside	9.17 ± 0.17	nd

[a]nd, not detected. [b]Determined in this study.

Cell Culture. Human gingival fibroblasts (HGF-1) (ATCC CRL-2014), used as the oral epithelial model in this study, were cultured in high glucose Dulbecco's modified Eagle's medium (DMEM) (Lonza, Basel, Switzerland) supplemented with 1% (v/v) penicillin/streptomycin solution (1:1) (Sigma-Aldrich, St. Louis, MO) and 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Biowest, Nuaillé, France). Cells were maintained at 37 °C in 5% CO_2 atmosphere and media was renewed every 3 days. Fibroblasts were subcultured once per week with a split ratio of 1:3 using 0.25% trypsin-EDTA solution (Sigma-Aldrich, St. Louis, MO).

Antimicrobial Study. Stock solutions of extracts and compounds mentioned above were prepared at 2 mg/mL final concentration in 4% dimethyl sulfoxide (DMSO) BHI or TSB media, depending on bacterial strain. Then, stock solutions were filtered (0.22 μM, Symta, Spain) and diluted to 1000, 500, 200, 100, 50, 25, 10 μg/mL final concentration. Microtitle assay[21] was carried out in a 96-wells plate, and 100 μL of each phenolic extract/compound was added to the pertinent well. Then, 100 μL of bacterial inoculum at 10^6 CFUs/mL final concentration was added. Negative (culture media without any inoculum/phenolic compound) and positive controls (bacteria without

DOI: 10.1021/acs.jafc.7b05466
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any treatment) as well as blanks (phenolic compounds dissolved in the culture media) were used to ensure the adequacy of the assay. A measurement ($O.D._{600\ nm}$) as $t = 0$ absorbance was taken on a Multiskan FC plate reader (Thermo Scientific). The microplate was incubated according to each strain requirements for 24−42 h at 37 °C under aerobic, anaerobic, or 5% CO_2 conditions, and absorbance was measured at selected intervals during 24 h, in order to determine the bacterial growth along time. MIC (minimum inhibitory concentration) and MBC (minimum bactericidal concentration) parameters were calculated and confirmed by microbial plate counting on BHI or modified TSB agar media. Assays were carried out in triplicate and three independent experiments were performed.

Cytotoxicity Assay. Cytotoxic effect of phenolic extracts and compounds at 50 and 10 μg/mL final concentration on HGF-1 fibroblasts viability was explored using the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were seeded on 96-wells plate cells 24 h prior to the assay. Then, complete culture media was replaced by compounds/extracts dissolved in cell culture media without FBS. Plate was incubated for 30 min or 24 h, and then media was replaced by MTT reagent diluted on sterile Dulbecco's Phosphate-Buffered saline (DPBS) solution (Lonza, Basel, Switzerland) (2 mg/mL). After 3 h incubation, MTT reagent was removed and ethanol−DMSO (1:1) mixture was added to dissolve formazan crystals. Absorbance was then measured at 570 nm on a Multiskan plate reader (Thermo Scientific). The absorbance ratio between cell culture treated with phenolics and the untreated control multiplied by 100 represents cell viability (percentage of control, %).

Bacterial Adherence Assay. *F. nucleatum*, *P. gingivalis*, and *S. mutans* were separately grown in their respective optimal conditions and all assays were carried out with single species bacterial cultures. Bacterial cells from an overnight culture ($O.D._{600\ nm} = 1$) were harvested by centrifugation (4500 rpm, 10 min at 4 °C) and resuspended in sterile DPBS solution. Fibroblasts were seeded on 48-well plates 24 h prior to the assay at a density of 2×10^5 CFUs/mL. The cell monolayer was washed twice with DPBS to remove any FBS or antibiotic residue, and 0.5 mL of a mixture of pure specie bacterial suspension at 10^7 CFUs/mL final concentration plus phenolic extracts, pure compounds or DPBS solutions was added to each well of HGF-1 cells (100:1 ratio of bacteria per fibroblast, in agreement with previous works[22]). After 30 min of incubation, supernatants were removed and cells were washed twice with DPBS in order to remove unbound bacteria. Then, cells and bound bacteria were detached with 0.25% trypsin−EDTA solution, and the reaction was stopped by adding cold DPBS. Bacterial counts were carried out on BHI agar plates for *S. mutans* and *S. dentisani* after 24 h of incubation while counts for other pathogens were carried out in TSB agar plates, after 48 h (*F. nucleatum*) or 5 days (*P. gingivalis*) of incubation. *S. mutans* and *S. dentisani* presented differences in the morphology of their colonies, which makes it possible to clearly differentiate them during counting. Adhesion capacity was calculated as the number of adhered bacteria (CFUs/mL) relative to the total number of bacteria initially added (% adhesion = [adhered bacteria/total bacteria] × 100). Results are expressed as percentage of adherence inhibition (%), calculated as 100(1 − T1/T2), where T1 and T2 are the number of oral pathogens adhered in the presence and absence of phenolics, respectively. Assays were performed in triplicate, and three independent experiments were carried out.

Antiadherence Assays: Exclusion, Competition, and Displacement of *S. mutans* by *S. dentisani*. *a. Adherence Exclusion.* For the exclusion assay, 0.5 mL per well of the mixed solution of *S. dentisani* (~10^7 CFUs/mL) and phenolic compounds or extracts were first added. After 15 min of incubation, nonbound bacteria were removed, 0.5 mL per well of *S. mutans* suspension (~10^7 CFUs/mL) was added, and the mixture was incubated again for 15 min. Cells together with bound bacteria were then detached with trypsin−EDTA solution, and the number of oral bacteria adhered to gingival cells was determined on BHI agar plates, as described above. Results are expressed as percentage of adherence inhibition (%), calculated as 100(1 − T1/T2), where T1 and T2 are the number of oral pathogens adhered in the presence and absence of *S. dentisani* and phenolics, respectively. Assays were performed in triplicate, and three independent experiments were carried out.

b. Adherence Displacement. Ability of *S. dentisani* and phenolics to displace previously adhered *S. mutans* was assessed as described above but first adding *S. mutans* suspension (~10^7 CFUs/mL) and later, *S. dentisani* suspension (~10^7 CFUs/mL) plus phenolic extracts and compounds. Displacement of pathogen was expressed as percentage of adhesion (%) of oral pathogen in the presence and absence of *S. dentisani* and phenolics, as described above.

c. Adherence Competition. The same experimental protocol as in exclusion was employed for the assays of competitiveness between *S. dentisani* and *S. mutans*, with the difference that oral pathogen (~10^7 CFUs/mL) and *S. dentisani* probiotic strain (10^7 CFUs/mL) plus phenolics were simultaneously added. Competitiveness was calculated as the percentage of adhesion (%) of *S. mutans* in combination with *S. dentisani* and phenolics relative to pathogen adhesion in the absence of *S. dentisani* and phenolic compounds (control).

UHPLC−DAD-ESI-TQ MS Targeted Analysis of Phenolic Metabolites. Incubations of cell monolayers with phenolic compounds (50 μg/mL) in absence (cellular metabolism) or presence of oral pathogens (bacterial and cellular metabolism) were carried out as mentioned above in three independent assays. After 30 min of incubation at controlled conditions, supernatants were collected and an aliquot was immediately frozen away (−80 °C) until further UHPLC−MS/MS analysis. Incubations only with phenolic compounds (blanks) or oral pathogens (bacterial metabolism) were also carried out.

Prior to their analysis, cellular supernatants were filtered (0.22 μM, Symta, Spain), and 200 μL of sample was mixed with 50 μL of internal standard (4-hydroxybenzoic-2,3,5,6-tetradeutered acid) (IS) prepared in acetonitrile and 0.1% formic acid at 0.25 μg/mL final concentration. A volume of 2.0 μL of sample was injected onto the chromatographic system. Analysis of each sample was performed in duplicate.

Phenolic metabolites were analyzed using an UPLC−ESI-MS/MS following a method previously published[23] with some modifications. 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 mm × 100 mm and 1.7 μm particle size from Waters (Milford, MA). The mobile phases were 0.1% formic acid in water (A) and 0.1% formic 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; 16.6 min, 99.9% B; 16.7 min, 0.1% B; 22 min 0.1% B. Equilibrium time was set at 2.4 min, resulting in a total runtime of 22.4 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 54 phenolic compounds targeted in the present study (hydroxyl(phenyl)-propionic, hydroxy-(phenyl)-acetic, hydroxycinnamic, hydroxybenzoic, and hydroxymandelic acids and flavan-3-ols) were previously determined.[23] All metabolites were quantified using the calibration curves of their corresponding standards. Data acquisition were carried out in multiple reaction mode (MRM) using transition of parent and product specific ions for each compound as well as by using the internal calibration curves. Data processing was performed with MassLynx v4.1 software (Waters) and results are presented as final concentration (μg/L) of the sample.

Statistical Analysis. Data obtained were submitted to statistical analysis in GraphPad Software v6.0 (GraphPad). Two-way ANOVA of multiple comparisons was applied to data of the study of the inhibitory effect against pathogenic adherence, followed by posthoc Dunnet test.

DOI: 10.1021/acs.jafc.7b05466
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Table 2. Antimicrobial Activity of Phenolic Compounds and Grape Seed and Red Wine Phenolic Extracts

	MIC/MBC (μg/mL)			
microorganisms	caffeic acid	*p*-coumaric acid	Vitaflavan	Provinols
S. mutans	>1000/>1000	>1000/>1000	>1000/>1000	>1000/>1000
P. gingivalis	200/500	500/1000	500/1000	500/>1000
F. nucleatum	1000/>1000	200/500	500/1000	500/1000
S. dentisani	1000/>1000	>1000/>1000	>1000/>1000	>1000/>1000

Data of UHPLC−MS/MS from the study of phenolic metabolism (three values from three independent assays, each value corresponding to the mean of the duplicate analysis of each sample) was analyzed by one-way ANOVA test followed by Bonferroni test. A value of $p < 0.05$ was fixed for the level of significance of the tests.

RESULTS

Antimicrobial Activity. Study of antimicrobial activity of phenolics against oral bacteria was assessed in order to select a concentration under the MIC for all compounds, for antiadherence assays (Table 2).[21] Phenolic compounds and extracts only showed antimicrobial activity at higher concentrations (above 200 μg/mL), and consequently, it was possible to use concentrations in the range normally found in wine, which is 0.3−33 mg/L for caffeic acid and 0.1−8 mg/L for *p*-coumaric acid.[24] Therefore, selected concentrations for the next assays were 10 and 50 μg/mL for each oenological extract and pure phenolic compound.

Cytotoxic Effect. With regards to a possible cytotoxic effect of compounds at selected concentrations on human fibroblasts, none of the phenolic compounds or extracts altered cell viability at 10 μg/mL nor 50 μg/mL (Figure 1) after 30 min of pretreatment but neither after 24 h incubation with compounds and extracts.

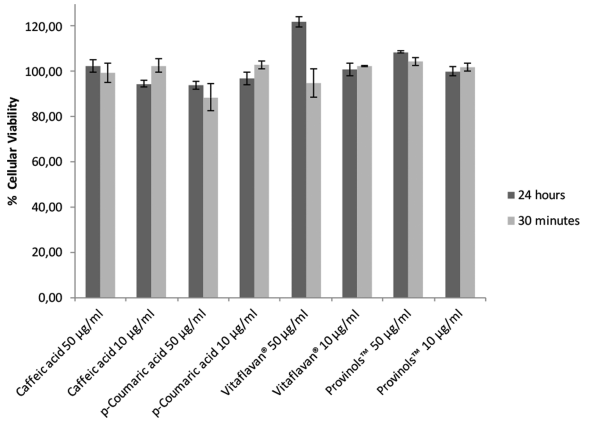

Figure 1. Cellular viability of HGF-1 fibroblasts in the presence of phenolic compounds and extracts (10 and 50 μg/mL) after 30 min and 24 h incubations. Results are expressed as media of three independent assays ± standard deviation.

Antiadhesive Effects on Oral Pathogen Adherence to HGF-1 Human Fibroblasts. With respect to antiadhesive activity of phenolic compounds and extracts, a different effect was perceived depending on the strain, phenolic concentration, and compound (Figure 2). In general terms, pure phenolic compounds (caffeic and *p*-coumaric acids) showed a greater ability to inhibit *S. mutans* and *F. nucleatum* adherence than phenolic extracts, whereas a similar effect for both treatments was reported in the case of *P. gingivalis* adherence to human fibroblasts.

S. mutans pathogen is normally related to caries disease, and therefore the ability of this bacterium to adhere fibroblasts is limited, around 1% (data not shown). Despite this fact, caffeic acid and *p*-coumaric acid at 50 μg/mL concentrations were able to decrease *S. mutans* adherence 20% and 40%, respectively ($p < 0.001$). Both compounds had a similar effect at 10 μg/mL concentration, reaching an inhibition of around 20−30% ($p < 0.001$). However, phenolic extracts Vitaflavan and Provinols did not exert any inhibitory effect on *S. mutans* adherence, except for Vitaflavan (10 μg/mL) ($p < 0.05$), and even an increase of adherence was observed for the higher concentration (50 μg/mL) (values of adherence inhibition <0% are not represented).

Inhibition of *P. gingivalis* adherence was tested for all the assayed compounds, including both pure phenolics and extracts (Figure 2C). Phenolic compounds reached higher values of inhibition when added at 10 μg/mL concentration ($p < 0.001$), whereas grape seed and red wine phenolic extracts were able to exert a stronger effect when used at 50 μg/mL concentration ($p < 0.001$). Inhibitory effects of caffeic and *p*-coumaric acids were similar, in the range of 30−50%. Provinols, a red wine extract, showed higher inhibitory potential (∼40−50%) than grape seed extract (Vitaflavan) (∼20−40%). Finally, in the case of *F. nucleatum* adherence, compounds exerted a stronger inhibition of pathogen adherence (*p* values ranging from <0.05 to 0.01) than extracts, especially, in the case of *p*-coumaric acid ($p < 0.01$) which caused a marked inhibition, upper than 50%. Furthermore, Provinols (10 μg/mL, $p < 0.01$) inhibition values were again higher than those of Vitaflavan.

Previous studies highlighted the potential role of *S. dentisani* in oral diseases.[14] With the aim of studying a possible complementary effect between this candidate oral probiotic and red wine phenolic compounds against *S. mutans* adherence to human fibroblasts, assays of inhibition of bacterial adherence were performed (Figure 3). Results confirmed a complementary action of both caffeic acid and *p*-coumaric acid, and *S. dentisani* in the competence process against *S. mutans* adhesion ($p < 0.05$), improving values from 25% to 40−50% in the presence of the coincubation of *S. dentisani* and phenolics. In the same way, exclusion was improved when caffeic acid was used together with the probiotic strain ($p < 0.05$), reaching values of 30−40% from the initial 10%. However, phenolic compounds were not able to improve the high displacement values of *S. mutans* (close to 80%) when only *S. dentisani* was used.

Bacterial and Cellular Metabolism of Oenological Extracts and Phenolic Compounds. Results of bacterial and cellular metabolism from UHPLC−MS analysis (Tables 3−5) showed that the three pathogens studied (*P. gingivalis*, *S. mutans*, and *F. nucleatum*) were able to release different phenolic metabolites after their incubation with phenolic compounds and extracts. Additionally, evidence also indicates an existing cellular metabolism of polyphenols by fibroblasts, which in some cases resulted in a cumulative metabolism between oral bacteria and human cells.

Phenolic metabolism in the absence or presence of fibroblasts and *P. gingivalis* is presented in Table 3. Regarding

DOI: 10.1021/acs.jafc.7b05466
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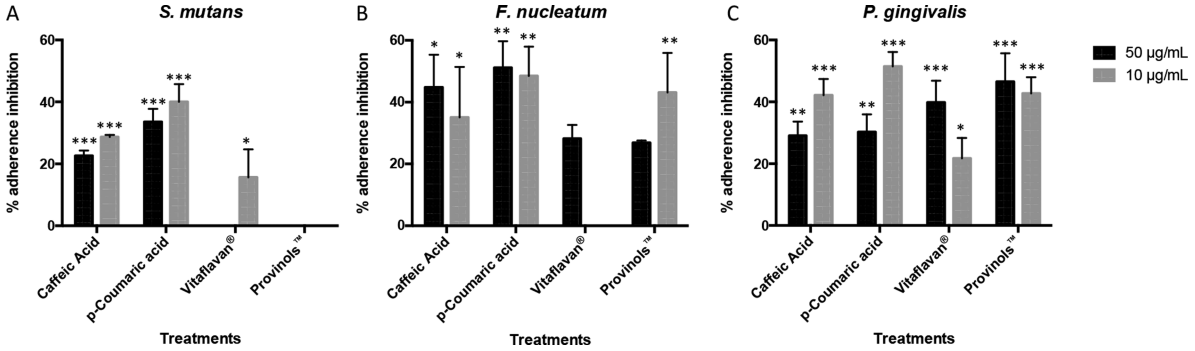

Figure 2. Adherence inhibition (%) of oral pathogens (A) *S. mutans*, (B) *F. nucleatum*, and (C) *P. gingivalis*) by phenolic compounds and extracts (10 and 50 μg/mL). Results are expressed as the media of three independent assays ± standard error. * indicates $p < 0.05$, ** indicates $p < 0.01$, and *** indicated $p < 0.001$.

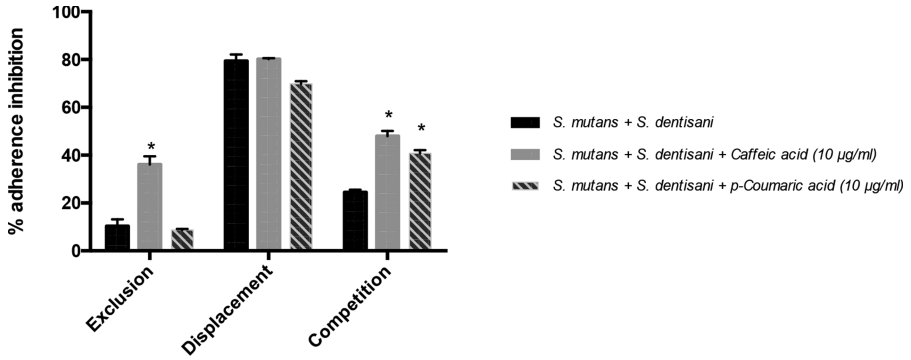

Figure 3. Percentage (%) of exclusion, displacement and competition of *S. mutans* adherence by *S. dentisani* probiotic strain 7746 and polyphenols. Results are expressed as the media of three independent experiments ± standard error. Data were analyzed by two-way ANOVA and Bonferroni test. * indicates $p < 0.05$.

oenological extracts, some compounds underwent thorough extensive metabolism during incubations. For instance, a significant degradation of gallic acid ($p < 0.01$) was found in the case of Vitaflavan after bacterial incubations, as in a similar manner that occurred in the case of proanthocyanidin B1 ($p < 0.05$) and Provinols, which reinforces the role of microbial catabolism of oligomeric tannins.

Regarding bacterial and cellular degradation of phenolic acids derived from the B-ring, 3,5-dihydroxybenzoic acid was metabolized reaching values close to 30% of degradation in the case of both oenological extracts, being especially significant in the case of Provinols ($p < 0.01$). In regard with nonflavonoids compounds, there was a bacterial degradation of protocatechuic acid, which was significant in the case of the incubation of *P. gingivalis* with Provinols extract ($p < 0.01$).

Additionally, 4-hydroxyphenyl acetic acid was detected only after incubations of all oenological extracts and phenolic compounds with *P. gingivalis* (ranging p values from <0.05 to 0.01), which highlights the potential of this bacteria to synthesize it, most likely from the catabolism of flavonols precursors.

Concerning phenolic metabolism in the presence of *S. mutans* (Table 4), a strong bacterial metabolism was observed for flavan-3-ols (+)-catechin ($p < 0.01$ to $p < 0.001$) and (−)-epicatechin ($p < 0.01$−0.001) after incubations with Vitaflavan. In a similar manner, levels of gallic acid strongly decreased after incubation of this pathogen with both extracts ($p < 0.01$−0.001) and also when cells or both, cells and bacteria, were present. Procyanidins B1 and B2 were markedly metabolized from Vitaflavan when cells and bacteria were simultaneously present ($p < 0.001$), whereas procyanidin B2 was increased in the presence of cells after treatment with Provinols ($p < 0.05$). In the same way, non-flavonoid protocatechuic acid from Provinols was significantly degraded after bacterial and cellular coincubations in the case of both extracts (p value ranging from $p < 0.05$ to $p < 0.001$). In relation to incubations of caffeic acid with *S. mutans*, a significant degradation of this compound was found ($p < 0.01$) only due to cumulative activity of *S. mutans* and HGF-1 cells.

Finally, data from phenolic metabolism in the absence or presence of fibroblasts and *F. nucleatum* assays were presented in Table 5. Metabolic activity of this bacterium seemed to be lower than the other two pathogens analyzed during this work. *F. nucleatum* was able to release gallic acid through bacterial catabolism ($p < 0.001$) from Vitaflavan extract. On the contrary to other pathogens, levels of *p*-coumaric acid from Vitaflavan significantly decreased after incubations with *F. nucleatum* ($p < 0.05$) or cells ($p < 0.01$). In accordance with results obtained from the other bacteria, levels of *p*-coumaric acid remained stable after incubations with the three pathogens, which resulted in a notable interest.

DISCUSSION

Previous studies discarded an antimicrobial effect of phenolic compounds and oenological extracts against gut microbial strains or respiratory pathogens in the range normally found in wine.[25,26] However, their effect on oral bacteria or on oral cells viability was unknown, and therefore, antimicrobial and cytotoxic effects were explored in the current work (Table 2, Figure 1). Antimicrobial activity of caffeic acid at high concentrations has been previously demonstrated.[24−26] However, concentrations used in our study (10 and 50 μg/mL) are not high enough to exert an

DOI: 10.1021/acs.jafc.7b05466
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Table 3. Phenolic Metabolite Concentration (μg/L) After Incubations in Absence or Presence of *P. gingivalis* and/or of HGF-1 Cells[a]

	Vitaflavan				Provinols				caffeic acid				*p*-coumaric acid			
metabolite	blank	bacterium	cells	bacterium + cells	blank	bacterium	cells	bacterium + cells	blank	bacterium	cells	bacterium + cells	blank	bacterium	cells	bacterium + cells
Phenolic Acids																
gallic acid	884.18 ± 12.48	533.82 ± 51.72**	754.00 ± 115.46	663.24 ± 142.11	108.07 ± 20.38	91.77 ± 8.73	133.79 ± 51.01	96.81 ± 15.13	–	–	–	–	–	–	–	–
protocatechuic acid	60.79 ± 4.11	45.25 ± 5.98	37.85 ± 4.49	41.72 ± 15.87	20.45 ± 6.81	13.53 ± 4.23**	33.62 ± 4.49	15.65 ± 6.34*	–	–	–	–	–	–	–	–
3,5-dihydroxybenzoic acid	62.27 ± 3.74	45.00 ± 5.66	33.50 ± 7.78*	44.67 ± 10.02	29.46 ± 4.64	14.67 ± 1.15**	22.89 ± 10.21**	14.33 ± 3.51**	–	–	–	–	–	–	–	–
4-hydroxyphenyl acetic acid	33.26 ± 0.01	389.28 ± 152.16*	–	418.62 ± 0.141.06*	–	399.06 ± 85.27*	–	522.95 ± 166.37*	–	359.94 ± 58.69*	–	366.46 ± 81.44*	–	412−10 ± 138.78*	–	425.14 ± 68.70*
3-(4-hydroxyphenyl) propionic acid	–	–	–	–	–	–	–	–	–	–	–	–	14215.21 ± 2052.15	14686.16 ± 555.20	15593.41 ± 555.20	14484.549 ± 3078.04
caffeic acid	–	–	–	–	–	–	–	–	57839.67 ± 1132.05	58879.37 ± 11895.37	69838.04 ± 7391.33	55997.27 ± 2718.09	–	–	–	–
p-coumaric acid	67.18 ± 17.94	33.06 ± 13.56*	17.33 ± 5.37**	34.21 ± 13.04*	37.26 ± 10.13	30.38 ± 3.84	31.14 ± 1.08	36.26 ± 3.10	–	–	–	–	25074.57 ± 460.25	23621.28 ± 2619.82	25232.70 ± 1974.38	23430.21 ± 2493.78
Flavan-3-ols																
(+)-catechin	3458.11 ± 84.41	3320 ± 237.15	3941.37 ± 414.31	3556.73 ± 771.64	227.94 ± 22.11	204.83 ± 49.66	415.15 ± 200.52	282.51 ± 42.92	–	–	–	–	–	–	–	–
(−)-epicatechin	2876.67 ± 162.40	2266.12 ± 616.34	3362.81 ± 713.87	2949.40 ± 602.27	208.72 ± 84.10	191.39 ± 27.13	189.48 ± 8.12	189.48 ± 26.31	–	–	–	–	–	–	–	–
procyanidin B1	1718.29 ± 0.125.84	1508.65 ± 159.78	1823.56 ± 326.68	1685.749 ± 205.02	363.75 ± 40.66	206.57 ± 34.78*	345.99 ± 86.58	265.87 ± 70.82*	–	–	–	–	–	–	–	–
procyanidin B2	973.33 ± 82.72	758.29 ± 148.29	1152 ± 71.39	927.90 ± 116.80	119.99 ± 26.56	126.25 ± 14.40	187.34 ± 64.02	115.05 ± 38.50	–	–		–	–	–	–	

[a]Data are expressed as mean values ± standard deviations. Data were analyzed by two-way ANOVA followed by the Bonferroni test. The * indicates values $p < 0.05$, ** indicates values $p < 0.01$, and *** indicates values $p < 0.001$; − means not detected.

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DOI: 10.1021/acs.jafc.7b05466

Table 4. Phenolic Metabolite Concentration (μg/L) After Incubations with *S. mutans*[a]

	Vitaflavan				Provinols				caffeic acid				*p*-coumaric acid			
metabolite	blank	bacterium	cells	bacterium + cells	blank	bacterium	cells	bacterium + cells	blank	bacterium	cells	bacterium + cells	blank	bacterium	cells	bacterium + cells
								Phenolic Acids								
gallic acid	884.18 ± 12.48	147.75 ± 27.15***	258.17 ± 41.27***	258.17 ± 41.27***	108.07 ± 20.38	16.56 ± 2.03***	25.77 ± 30.72***	6.20 ± 2.71***	–	–	–	–	–	–	–	–
protocatechuic acid	60.79 ± 4.11	54.411 ± 18.23	37.85 ± 4.49*	14.24 ± 5.32**	20.45 ± 6.81	16.35 ± 5.32*	22.89 ± 10.20	1.90 ± 0.50***	–	–	–	–	–	–	–	–
3-(4-hydroxyphenyl) propionic acid	–	–	–	–	–	–	–	–	–	–	–	–	14215.21 ± 2052.15	15831.65 ± 855.37	15211.61 ± 547.44	14067.54 ± 1211.78
caffeic acid	–	–	–	–	–	–	–	–	57839.67 ± 1132.05	69599.13 ± 2579.94	69838.04 ± 7391.33	27962.85 ± 3920.67**	–	–	–	–
p-coumaric acid	67.18 ± 17.94	15.28 ± 1.93**	47.76 ± 12.40	6.08 ± 2.34***	37.26 ± 10.13	134.70 ± 45.69**	40.85 ± 24.93	51.08 ± 20.30	–	–	–	–	25074.57 ± 460.25	24640.18 ± 1696.69	28867.96 ± 3155.56	28011.55 ± 3324.63
								Flavan-3-ols								
(+)-catechin	3458.11 ± 84.41	1349.29 ± 152.56**	3906.32 ± 695.37	2700.28 ± 456.68	227.94 ± 22.11	307.15 ± 75.05*	384.83 ± 8.04	108.19 ± 47.67*	–	–	–	–	–	–	–	–
(−)-epicatechin	2876.67 ± 162.40	1180.91 ± 109.60**	3459.46 ± 572.47	2629.77 ± 412.49	208.72 ± 84.10	271.78 ± 106.24	301.45 ± 60.90*	91.87 ± 32.48	–	–	–	–	–	–	–	–
procyanidin B1	1718.29 ± 0.125.84	2357.70 ± 415.06*	2067.05 ± 384.88	662.76 ± 92.79***	363.75 ± 40.66	403.73 ± 46.23	370.95 ± 224.51	156.77 ± 52.58	–	–	–	–	–	–	–	–
procyanidin B2	973.33 ± 82.72	1480.67 ± 267.96**	1278.23 ± 60.59	451.80 ± 115.02**	119.99 ± 26.56	187.00 ± 21.97	224.80 ± 67.72*	136.59 ± 14.26	–	–	–	–	–	–	–	–

[a]Data are expressed as mean values ± standard deviations. Data were analyzed by two-way ANOVA followed by the Bonferroni test. * indicates values $p < 0.05$, ** indicates values $p < 0.01$, and *** indicates values $p < 0.001$; − means not detected.

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DOI: 10.1021/acs.jafc.7b05466

Table 5. Phenolic Metabolite Concentration (μg/L) After Incubations with *F. nucleatum*.[a]

	Vitaflavan				Provinols				caffeic acid				*p*-coumaric acid			
metabolite	blank	bacterium	cells	bacterium + cells	blank	bacterium	cells	bacterium + cells	blank	bacterium	cells	bacterium + cells	blank	bacterium	cells	bacterium + cells
								Phenolic Acids								
gallic acid	884.18 ± 12.48	1667.51 ± 03.40***	754.00 ± 115.46	2130.56 ± 108.77***	108.07 ± 20.38	135.47 ± 15.40	133.79 ± 51.00	214.05 ± 13.26***	–	–	–	–	–	–	–	–
3-(4-hydroxyphenyl) propionic acid	–	–	–	–	–	–	–	–	–	–	–	–	14215.21 ± 2052.15	17307 ± 3024.75	1482056 ± 1023.07	16803.10 ± 2328.03
3,5-dihydroxybenzoic acid	62.27 ± 3.74	37.85 ± 16.45	33.62 ± 7.48	49.48 ± 13.20	29.46 ± 4.64	12.47 ± 1.50	22.89 ± 10.21	30.45 ± 11.58								
caffeic acid	–	–	–	–	–	–	–	–	57839.67 ± 1132.05	68001.98 ± 4992.38	71072.47 ± 2413.96	65881.80 ± 2760.38	–	–	–	–
p-coumaric acid	67.18 ± 17.94	38.04 ± 12.81*	17.33 ± 5.37**	53.40 ± 3.52	37.26 ± 10.13	25.00 ± 4.80	31. 14 ± 1.08	41.12 ± 6.28	–	–	–	–	25074.57 ± 460.25	28203.84 ± 3086.01	24505.26 ± 972.74	28818.61 ± 4431.67
								Flavan-3-ols								
(+)-catechin	3458.11 ± 84.41	3033.77 ± 418.03	3941.37 ± 414.31	2817.76 ± 440.73	227.94 ± 22.11	244.62 ± 67.35	415.15 ± 200.52	278.25 ± 92.52	–	–	–	–	–	–	–	–
(−)-epicatechin	2876.67 ± 162.40	3313.05 ± 341.05	2951.31 ± 56.84	2629.77 ± 412.49	208.72 ± 84.10	139.72 ± 44.60	189.48 ± 8.12	262.21 ± 28.90*	–	–	–	–	–	–	–	–
procyanidin B1	1718.29 ± 125.84	1455.77 ± 17.00	1823.56 ± 326.68	1435.34 ± 232.38	363.75 ± 40.66	241.83 ± 105.44	345.99 ± 86.58	247.84 ± 69.60	–	–	–	–	–	–	–	–
procyanidin B2	973.33 ± 82.72	1174.52 ± 129.18	1152.08 ± 84.69	1281.20 ± 171.45*	119.99 ± 26.56	80.77 ± 18.00	187.34 ± 64.02	127.85 ± 11.09	–	–	–	–	–	–	–	–

[a]Data are expressed as mean values ± standard deviations. Data were analyzed by two-way ANOVA followed by the Bonferroni test. * indicates values $p < 0.05$, ** indicates values $p < 0.01$, and *** indicates values $p < 0.001$; – means not detected.

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DOI: 10.1021/acs.jafc.7b05466

antimicrobial effect against oral pathogens, and therefore, it makes it possible to study other mechanisms of action, such as the bacterial antiadhesive effect. For instance, caffeic acid was previously described to possess antimicrobial activity against *S. mutans* (2 mg/mL),[27] and an inhibition of cariogenic *S. mutans* and *S. sobrinus* growth by a propolis extract with elevated content of both caffeic and *p*-coumaric acids was reported.[28] In a similar manner, antimicrobial activity of caffeic acid (156.3 μg/mL) against pathogenic strains resistant to conventional antibiotics (*Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*) was observed.[29] Recent evidence suggested that antimicrobial action of caffeic acid (and other phenolics, such as gallic acid and proanthocyanidins) against *S. mutans* could be based on the ability of polyphenols to generate hydroxyl radicals which would produce H_2O_2 and subsequent damage in bacterial DNA.[30] Antimicrobial effect of dietary polyphenols against periodontal pathogens has been explored too. Both grape seed and red wine extracts (>1000 μg/mL) reduced the counts of *P. gingivalis* and *F. nucleatum*,[31] whereas Muñoz-González and colleagues reported a bactericidal action of caffeic acid from red wine (1600 μg/mL) and grape seed extract (250 μg/mL) against *F. nucleatum*.[18] Polyphenols from different berries have been also described to inhibit *F. nucleatum* (>63 μg/mL) and *S. mutans* (>16 μg/mL).[32] However, high ranges employed on the studies mentioned above are far from physiological levels, and so other mechanisms of action should be explored. Finally, red wine was recently reported to display an antimicrobial action in an oral biofilm model containing *Actynomyces oris*, *F. nucleatum*, *Streptococcus. oralis*, *S. mutans*, and *Veillonella dispar* as well as against selected oral streptococci.[18,33]

A key step in bacterial infection is pathogenic adhesion to host cells,[34] and an antiadhesion therapy is an efficient way to prevent or treat bacterial infections. The percentage of adhesion of *S. mutans* control to human fibroblasts was around 1%, which is relatively low when compared to other species. Despite of this, our results showed that *S. mutans* adhesion to HGF-1 human fibroblasts was partially inhibited after treatment with caffeic (∼20−25%) and *p*-coumaric (∼35−40%) acids (Figure 2A). A similar study with phenolic acids of pu-erh and chrysanthemum tea enriched in tannins reported an inhibition of *S. mutans* attachment to oral cells and, in the same line, control adherence was around 1%.[22] *S. mutans* is a cariogenic pathogen and therefore is generally found on solid surfaces. Because of this, the most part of the studies regarding inhibition of bacterial attachment are carried out in solid surfaces that mimic human teeth.[35] However, last evidence suggested that it also inhabits soft oral tissues.[36] For instance, the antiadhesive effect of dealcoholized red wine over *S. mutans* in teeth was described,[37] and this fact was allocated to procyanidins, high weight tannins that could act as stearic impediment for *S. mutans* attachment. In cellular surfaces, this merge is mediated through specific receptors and bacterial attachment to cells should be high enough to facilitate the injection of bacterial virulent proteins into host cells.[34] No effect was perceived for the extracts and, in agreement with our results, a previous study carried out in a 5-species oral biofilm, including *S. mutans*, with Vitaflavan and Provinols reported that none of these extracts affected *S. mutans* viability.[18] Concerning *F. nucleatum* adherence, an inhibitory action of caffeic and *p*-coumaric acids and Provinols extract was observed (Figure 2B). In accordance, it is known that *F. nucleatum* adheres to a selected range of human cells, including fibroblasts and a recent study demonstrated an antiadhesive action of a green tea extract, epigallocatechin, and theaflavins against *F. nucleatum* adherence to oral epithelial cells.[38] The role of "physical bridge" described for this bacterium makes of the antiadhesive therapy a useful strategy to avoid periodontal diseases since it favors the attachment of other pathogens. As an example, *F. nucleatum* biofilm formation is stimulated by *P. gingivalis* due to the expression of signaling molecules,[39] as well as *F. nucleatum* protects *P. gingivalis* against aerobic conditions, by generating a capnophilic environment.[40] Therefore, the inhibition of *F. nucleatum* by phenolic compounds alone or in combination with *S. dentisani* is particularly relevant because this species has been shown to be a key player in biofilm architecture, and many periodontal pathogens use it as an attachment.[41] Thus, inhibition of *F. nucleatum* adhesion or growth has been proposed as a promising strategy to reduce plaque formation and prevent the settlement of oral pathogens.[42]

Our results showed that *P. gingivalis* adhesion to human fibroblasts was inhibited by all the assayed extracts and compounds (Figure 2C). In accordance with these observances, a high-molecular-weight fraction from cranberry, enriched in proanthocyanidins, prevented *P. gingivalis* attachment to surfaces coated with collagen, fibrinogen, and human serum,[43] whereas A-type proanthocyanidins from the same food source inhibited *P. gingivalis* adhesion to oral cells.[44] Furthermore, these compounds were able to prevent teeth demineralization during the cariogenic process.[45] Beneficial actions of resveratrol against periodontal diseases have been associated with its ability to neutralize *P. gingivalis* pathogenic factors.[46,47] In a similar manner, a green tea extract inhibited *P. gingivalis* attachment to buccal cells,[48] and also resveratrol (1−10 μM) blocked the expression of adhesion proteins of *P. gingivalis*.[46] In our study, resveratrol was present in red wine extract, which is in accordance with the higher inhibition of adherence observed after incubation with Provinols. In general terms, a dose-dependent inhibitory effect of pathogenic adherence was not perceived. One of the mechanisms that have been suggested for the antiadhesive potential of polyphenols is that they constitute a stearic impediment for bacterial attachment to cellular receptors.[49] There is a limited number of surface receptor of mammalian cells for bacterial adhesins, and therefore, a maximum inhibitory effect of polyphenols is going to be achieved at a specific certain concentration, and therefore, an increase in the phenolics concentration may not be necessarily translated into more effectiveness.

S. dentisani presented a similar resistance to the antimicrobial potential of the selected polyphenols to *S. mutans* pathogen, as shown in Table 2. This fact makes possible a combinatory study of the preventive action of wine polyphenols and this candidate oral probiotic. After coincubations with *S. dentisani* strain and phenolic compounds, a cumulative antiadhesive effect on *S mutans* was perceived (Figure 3). A reciprocal action between polyphenols and probiotics has been previously reported in the case of red wine phenolic extract and gut probiotic strains (e.g., *Lactobacillus* and *Bifidobacterium*[50] and also oenological-origin probiotic strains, which improved inhibitory effects of Provinols against *E. coli* adhesion to intestinal cells.[51] It is a two way interaction: probiotics are able to improve polyphenols bioavailability,[52] whereas gut microbiota is modulated by dietary polyphenols.[2,53] Our results showed that such reciprocal effect was marked for both compounds in the case of adherence competition, which suggests that polyphenols could improve the competition of *S. dentisani* candidate oral probiotic strain against *S. mutans* for cellular receptors. Additionally, a significant effect was observed with caffeic acid on the of *S. mutans* adherence exclusion but not on its adherence displacement, which was

DOI: 10.1021/acs.jafc.7b05466
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probably due to *S. mutans* being first attached to fibroblasts. These evidence highlight the potential of grape derived polyphenols as natural therapy to prevent caries and periodontal diseases, alone or in combination with traditional treatments. However, the effects mostly depend on particular combinations of phenolic structures, and it would be helpful to clarify the mechanisms of action involved on the prevention of oral diseases as well as determine the phenolic metabolism that take place *in vivo*, in order to design an effective strategy.

Dietary polyphenols go through several modifications along the digestive system before reaching systemic circulation, and recent evidence suggested that real executors of benefits implied from intake of dietary polyphenols are the metabolites rather than the parent compounds.[2] The most part of this phenolic metabolism takes place in the gut; however, these transformations start in the oral cavity, where dietary components suffer mechanical and chemical alterations.[2] As far as we are aware, only a little evidence has been focused on the study of oral metabolism of polyphenols,[18,54,55] and previous findings confirmed the ability of probiotic strains to metabolize oenological extracts releasing phenolic metabolites that enhanced bacterial growth.[50] Our results confirmed for the first time the relevance of bacterial and cellular phenolic metabolism as well as a complementary metabolic action (Tables 3−5). This metabolism included the degradation of precursors into phenolic metabolites as well as other enzymatic reactions. A relevant effect was perceived for bacterial metabolism of proanthocyanidins, catechins, and epicatechins. Microbial catabolism of monomeric galloylated flavan-3-ols start with the fast scission of the ester group from gallic acid by microbial esterases, which leads to the generation of pyrogallol and the monomers (+)-catechin and (−)-epicatechin.[17] A significant degradation of gallic acid, (+)-catechin, and (−)-epicatechin for *S. mutans* and of gallic acid by *P. gingivalis* was observed whereas *F. nucleatum* released gallic acid through esterase activity. Degradation of gallic acid by *S. mutans* and *P. gingivalis* could suggest a deeper transformation of these compounds into some other bioactive derivatives, which would prevent bacterial adhesion. In accordance with these observances, previous studies of the antimicrobial properties of red wine and grape seed extracts in an oral biofilm model revealed a bacterial metabolism of flavan-3-ols precursors, as determined by UHPLC−MS/MS.[18] Procyanidins B1 and B2 were strongly degraded after incubation with *S. mutans* and cells, especially in the case of Vitaflavan which is in agreement with the release and subsequent degradation of gallic acid.

Differences in the ability of oral pathogens to metabolize phenolics were perceived. For instance, *S. mutans* degraded gallic acid independently from phenolic source, whereas the action of *P. gingivalis* over this compound is moderate, which might suggest a stronger esterase and decarboxylase activities of *S. mutans*. 4-Hydroxyphenylacetic acid, originated from sequential α-oxidations from (hydroxyphenyl)propionic acids, was only detected due to *P. gingivalis* metabolism. *p*-Coumaric acid metabolism rarely varied: it remained stable in all the incubations, standing out as a direct effector of the inhibition of the bacterial adhesion. On the contrary, *p*-coumaric acid from Vitaflavan become partially degraded by *P. gingivalis* and *S. mutans*. This suggests that the wide variety of bacterial functionalities observed *in vivo*, might be due to the existence of bacterial consortiums rather than to the activity of isolate species. In this context, phenolic metabolism would occur in a sequential manner, leading to the production of a huge variety of chemical structures, depending on the microbial ecology present.[56] Other limiting factor in phenolic metabolism is their structural and stereo chemical characteristics, which determines the bioaccessibility to phenolic substrate.[17] Additionally, a bottleneck that limits these studies is the individual variability, in microbial composition and in physiological parameters, such as salivary composition. It is also important to point out the contribution of cellular metabolism, rarely explored.[57] In our study, a degradation of 3,5-dihydroxybenzoic acid from Provinols was perceived for *P. gingivalis* but only in the presence of cells or coincubations, which suggest a bacterial and cellular ability to transform it into some other derivatives, such as vanillic or hydroxybenzoic acids. In agreement with our evidence, Mena and coworked recently demonstrated the role of cellular phenolic metabolism of flavan-3-ols in the inhibition of bacterial adhesion to bladder epithelial cells.[58] Also, caffeic acid was degraded by *S. mutans* in the presence of cells, highlighting the cellular hydrolytic activity, together with a collaborative action with the pathogen. This is in agreement with the oxidation of caffeic acid catalyzed by cellular enzymes, such as peroxidases and tyrosinases.[59] With regard to nonflavonoids compounds, the most common microbial metabolites of caffeic acid are hydroxyphenyl propionic and protocatechuic acids.[18] Protocatechuic acid (3,4-dihydroxybenzoic acid) was partially degraded only in the presence of *P. gingivalis*. Additionally, the content of this compound was significantly increased in the presence of *F. nucleatum*, whereas coincubations with cells improved its degradation.

Study of bacterial phenolic metabolism is of high relevance in order to determine which compounds are the real inhibitors of bacterial adhesion and design an antiadhesive strategy. Furthermore, in a condition of disease, an overgrowth of pathogenic bacteria is triggered and phenolic metabolism in plaque will be mainly due to pathogenic activity. At this point, LC−MS techniques constitute a useful approach, as shown in previous research. Walle and colleagues[54] reported a release of aglycones from flavonoid glycosides (e.g., quercetin, genistein) by microbial β-glucosidases and also hydrolytic activity of oral cells by using HPLC. A similar study with LC−MS/MS revealed a contribution of human oral epithelial tissue, salivary, and microbial enzymatic activity in phenolic metabolism of anthocyanidins.[60] Our study, based on an *in vitro* model of bacterial adherence results, is very useful as an initial approach to go deeper into the mechanisms of action of red wine polyphenols against oral diseases. Further steps should be addressed toward the use of mixed biofilms models which can mimic bacteria−bacteria interactions as well as some other conditioning factors that should be added to multifactorial assays. Once molecular mechanisms of action become elucidated, *in vivo* studies of periodontal and cariogenic diseases are recommended, in order to evaluate the potential of polyphenols as preventive therapies in the management of cariogenic and periodontal diseases.

AUTHOR INFORMATION

Corresponding Author

*E-mail: victoria.moreno@csic.es.

ORCID

M. Victoria Moreno-Arribas: 0000-0002-4136-595X

Funding

This work was funded by the Spanish MINECO (Grant AGL2015-64522-C2-R project) and Comunidad de Madrid (Grant ALIBIRD-CM S2013/ABI-2728).

Notes

The authors declare no competing financial interest.

DOI: 10.1021/acs.jafc.7b05466
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■ ACKNOWLEDGMENTS

A.E-F and I.Z-P are recipients of fellowships from the FPI-MINECO Programme.

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