Stability of saliva microbiota during moderate consumption of red wine

Elvira Barroso^{a§}, Virginia Martín^{b,c§}, M. Carmen Martínez-Cuesta^a, Carmen Peláez^a,
Teresa Requena^{a*}

*^aDepartment of Food Biotechnology and Microbiology, Institute of Food Science
Research, CIAL (CSIC-UAM), Nicolás Cabrera 9, 28049 Madrid, Spain*

*^bDepartamento de Nutrición, Bromatología y Tecnología de los Alimentos. Facultad de
Veterinaria, UCM, Av. Puerta de Hierro, 28040 Madrid, Spain*

^cProbisearch, Santiago Grisolia 2, Tres Cantos, 28760, Spain

*Corresponding author. Department of Food Biotechnology and Microbiology, Institute
of Food Science Research, CIAL (CSIC-UAM), Nicolás Cabrera 9, 28049 Madrid,
Spain. Tel.: +34 910017900; fax: +34 910017905.

E-mail address: t.requena@csic.es

§ Equal contribution

HIGHLIGHTS

- We evaluate the effect of red wine intake on human saliva microbiota
- Representative bacterial genera are common in saliva from all volunteers
- Inter-individual variability of saliva microbiota surpasses the wine intake effect
- Stability of the human saliva microbiota is not disturbed by red wine consumption

Abstract

Objective: This study has evaluated the effect of regular and moderate red wine consumption on the diversity and occurrence of different groups of bacteria that are representative in human saliva.

Methods: Saliva from twenty-two healthy volunteers (age range 20–48 years) was analyzed in this study. Fourteen individuals consumed red wine (250 mL/day) during 4 weeks, whereas 8 volunteers were included in the control group. The evolution and composition of the microbial community in saliva was evaluated by PCR-DGGE and quantitative PCR.

Results: The microbial inter-individual variability observed in the PCR-DGGE band patterns was higher than the differences observed after the 4-weeks period of red wine intake. *Bifidobacterium dentium*, *Bifidobacterium* spp. and *Alloscardovia omnicolens* were the most representative bifidobacterial species, whereas the *Streptococcus mitis*-*Streptococcus oralis* group predominated within *Streptococcus*. This genus was the most numerous of the bacterial groups assayed, reaching average counts above 8 log copy numbers/mL. On the other hand, the lowest counts were recorded for *Actinomyces*, *Fusobacterium*, *Haemophilus*, *Neisseria* and *Veillonella*, which showed average values of 5 log copy numbers/mL. The results showed no significant differences ($P>0.5$) in bacterial counts after the period of red wine intake.

Conclusion: The overall diversity and stability of representative bacterial groups of the human saliva is not disturbed due to regular-moderate red wine consumption.

Keywords: Saliva; Microbiota; Red wine; Homeostasis; *Streptococcus*

1. Introduction

Regular intake of polyphenol-rich beverages and foods has demonstrated to exert beneficial effects in human health, such as decreased incidence of cardiovascular disease, cancer and protection against neurodegenerative diseases, among others.^{1–3} Nevertheless, the beneficial effects of polyphenols seem to be more linked to microbial phenolic metabolites produced in the human gut than to the original forms present in food⁴. Accordingly, regular and moderate intake of red wine (a characteristic polyphenol-rich beverage) has demonstrated to exert modulating effects in the human gut microbiota.^{5–7} It is reasonable to presume that besides their effect on gut microbiota, these polyphenols can exert an effect on the overall oral cavity microbiota (saliva and gingival margins). This is worth considering that diversity of the microbial populations in the oral cavity is even larger than in the gut or the skin, harboring viruses, archaea, protozoa, fungi and over 700 species of bacteria.^{8,9} Similarly to human gut or skin microbiome, the oral microbial community is an interacting ecosystem with the host that helps to maintain the health status, although certain ecological shifts allow pathogens to establish and cause disease.^{10,11} Despite its relevance to human health, little information is currently available on the effect, if any, of diet on the saliva and gingival dental microbiota. A study on the salivary microbiota of individuals who followed an omnivore or ovo-lacto-vegetarian or vegan diet has recently been performed.¹² However, there is scarce information about the effect of daily habits such as regular red wine consumption on the oral cavity microbiota.

The antimicrobial effects of the polyphenols present in red wine and grape seed extracts against microorganisms responsible for periodontitis and dental caries have been mainly studied by incubating polyphenols with pure strains.^{13–16} Signorello et al.¹⁷ evaluated the microbial composition of supragingival and subgingival plaque in 75 adult volunteers that had been drinking 400 ml red wine daily for at least two previous years. It was observed a lower microbial diversity in the plaque samples of regular wine drinkers compared with water drinkers. This microbial modulating effect was speculatively attributed to the antimicrobial, antiadhesive, and antiplaque activities of the polyphenols contained in wine. In a review targeting bacterial adhesion to different substrates, the same authors considered the polyphenol-rich foods as a potential alternative strategy to antibiotic therapy in order to avoid caries and gingivitis/periodontitis.¹⁸

Concerning saliva, the effect of consumption of red wine on the antioxidant status of this oral fluid has been recently investigated,¹⁹ but information on the effect of red wine in the microbial community of saliva is still scarce. Saliva is crucial to oral cavity health, containing a characteristic bacterial community that helps maintaining homeostasis of the mouth ecosystem and it is considered useful in prognosis of several systemic and oral dysfunctions.^{11,20} Oral health is characterized by an ecologically balanced and diverse microbiome and the analysis of plaque and saliva in healthy adults has demonstrated larger microbial biodiversity than in people suffering symptoms of caries or periodontitis.¹¹ It is therefore essential to health the maintenance of oral cavity homeostasis through a well-balanced and stable oral microbiome in which saliva plays a key role.

Recent metagenomic studies have revealed that *Streptococcus*, *Veillonella*, *Neisseria*, *Prevotella* and *Fusobacterium* are among the most predominant microorganisms in the saliva of healthy individuals.^{21–23} Nevertheless, under various circumstances, oral homeostasis is lost and certain species from these and other genera, such as *Haemophilus*, *Lactobacillus* and *Porphyromonas*, turn out to be opportunistic and can represent a potential threat for both oral and respiratory tract health status.^{11,24,25}

The purpose of this work has been to study if the regular and moderate consumption of red wine during four weeks can affect somehow the diversity and occurrence of different groups and genera of bacteria that are representative in human saliva and whether it can influence the local microbial balance needed to keep oral health.

2. Materials and methods

2.1. Wine intervention study and sampling

Saliva samples were obtained during the human intervention study with moderate intake of red wine described by Muñoz-González et al.²⁶ Briefly, 22 healthy non-smokers volunteers (age range 20–48 years), included in the wine intervention trial (14 individuals) and in the control group (8 volunteers), provided saliva samples at the beginning of the study and after 4 weeks. The participants were not suffering from any oral disease and were not receiving antibiotics or any other medical treatment for at least 6 months before the start of the study. All participants were informed about the study objective and provided informed written consent. The study was approved by the

Ethics Committee of Hospital Ramón y Cajal (Madrid, Spain) and Spanish Council of Scientific Research (CSIC; Spain).

The study was preceded by a washout period of 2 weeks (baseline) during which individuals were asked to not consume any other alcoholic beverages and to follow a low-polyphenols diet (reduced intake of coffee, tea, cacao, berries, red fruits, etc.). After the washout period, the intervention group consumed daily 250 mL (consumed at lunch and dinner time during 4 weeks) of young red wine (Pinot Noir, vintage 2010), provided by Miguel Torres winery (Spain) and characterized by relatively high polyphenols content (1758 mg of gallic acid equivalents/L). During this period, volunteers were asked to not consume any other alcoholic beverages and to follow a low-polyphenols diet. The control group maintained the restriction for alcoholic beverages and recommendation for a low-polyphenols diet during the whole study.

Each participant provided two samples (after the washout period and at the end of the study) of saliva. Unstimulated whole saliva was collected at morning before teeth brushing and food or beverage consuming. Samples were placed in tightly covered sterile bottles and delivered to the laboratory in less than 2 h for treatment and analyses.

2.2. Sample preparation and DNA extraction

Saliva samples were centrifuged at 10000 $\times g$, 15 min at 4 °C, and pellets were stored at -80 °C with 40% glycerol. For DNA extraction, samples were thawed, mixed with 100 μ L sodium phosphate buffer, pH 7.0, and 100 mg of glass beads (150–212 μ m diameter). Cells were mechanically lysed by using a FastPrep equipment (BIO 101, Savant Instruments, Holbrook, NY) for 45 s (three times) at a machine speed setting of 5.5 m/s. The aqueous phase containing genomic DNA was separated by centrifugation, 10000 $\times g$ for 10 min at 4 °C, and DNA was extracted using the NucleoSpin Tissue kit (Macherey-Nagel, Düren, Germany).

2.3. PCR-DGGE

The diversity of the bacterial communities in the saliva samples and the effect of the red wine consumption were assessed by PCR-DGGE and using the primers and annealing temperatures described in Table 1. Samples were tested for total bacteria, *Streptococcus*, *Veillonella*, *Neisseria*, *Lactobacillus*, *Bifidobacterium* and the group *Prevotella*/*Porphyromonas*/*Bacteroides*. For *Lactobacillus* and *Bifidobacterium*, a

nested PCR step was included that was carried out as described by Heilig et al.²⁷ and Satokari et al.,²⁸ respectively. DGGE was performed with a DCode system (Bio-Rad) using a 9% polyacrylamide gel and a genus-specific gradient (Table 1) of 7 M urea and 40 % formamide. The obtained band patterns were analyzed using InfoQuest FP software version 5.1 (Bio-Rad). Selected DGGE bands were sequenced after excision from the gel and PCR reamplified with each specific primer set.

2.4. Quantitative PCR (qPCR)

Total bacteria and different groups and genera of bacteria that are representative in human saliva (Table 1) were quantified by qPCR using SYBR green methodology (Kappa Biosystems, Woburn, MA, USA) with the IQ5 Multicolor Real-Time PCR Detection System and data analyses (Bio-Rad Laboratories Inc., Hercules, USA). DNA from *Escherichia coli* DH5 α , *Lactobacillus plantarum* IFPL935, *Bifidobacterium lactis* Bb12 and *Streptococcus thermophilus* ATCC 19987 was used for quantification of total bacteria, *Lactobacillus*, *Bifidobacterium* and *Streptococcus*, respectively. For the rest of the groups analyzed (*Actinomyces*, *Fusobacterium*, *Haemophilus*, *Neisseria*, *Veillonella* and *Prevotella*), samples were quantified using standards derived from targeted cloned genes using the pGEM-T cloning vector system kit (Promega, Madison, WI, USA), as described previously by Barroso et al.²⁹

2.5. Statistical analysis

Mean values and standard deviations were calculated on the basis of the qPCR values for the different bacterial groups before and after the 4-week study period, for both the control and intervention groups. The Student *t*-test for dependent samples was used to evaluate differences in the counts of bacterial groups before and after the 4-week period. Statistical analyses were carried out using the IBM SPSS Statistics 21 package (SPSS Inc., Chicago, IL, USA).

3. Results and discussion

The potential changes in the diversity of bacterial groups after daily consumption of 250 mL of red wine during four weeks were analyzed by PCR-DGGE. The usefulness of applying DGGE to monitor temporal and developmental changes in oral bacterial

communities from saliva and dental plaque has been shown by Rasiah et al.³⁰ Results using primers targeting total bacteria showed no apparent changes in the band patterns due to red wine intake (Fig. S1, supplementary material). In general, the inter-individual variability in the band patterns was higher than the differences observed after the 4-weeks period of red wine intake.

Considering the observed differences in sensibility of specific oral microbial groups to wine and grape seed polyphenols,^{16,31} we evaluated the PCR-DGGE band profiles of representative oral bacterial groups before and after red wine intake. As an example, Fig. 1 shows the *Streptococcus* and *Bifidobacterium* fingerprints representative of eight individuals before and after red wine intake. Results from samples of the individuals distinguished in the *Bifidobacterium* PCR-DGGE patterns three main bands that, after nucleotide sequencing, were identified to correspond with *Bifidobacterium dentium*, *Bifidobacterium* spp. and *Alloscardovia omnicolens* (Fig. 1a). This last species has recently been described to belong to the *Bifidobacteriaceae* family³² and to be associated with human saliva³³. Again, more differences in the *Bifidobacterium* fingerprints were observed between individuals than over the period of red wine intake in each individual. Similar results were observed following the specific PCR-DGGE analysis of *Streptococcus* (Fig. 1b). In this case, the predominant band was sequenced and identified as belonging to the *S. mitis*-*S. oralis* group. *S. mitis* is the most common bacterium isolated from the oral cavity (Stahringer et al., 2012). Regarding the rest of bacterial genera examined, no consistent PCR-DGGE fingerprint differences associated to red wine intake were either observed for *Lactobacillus*, *Veillonella*, *Neisseria* or *Prevotella*-*Porphyromonas*-*Bacteroides* group (results not shown).

A complementary approach to evaluate the effect of red wine intake was the evaluation by qPCR of the potential quantitative changes of representative bacterial groups present in the oral cavity (Table 2). Total bacterial counts in saliva showed an average of 8 log copy numbers/mL in all individuals before and after the timeframe of the study. These figures are within the range of 10⁷ to 10⁹ bacteria per mL described for human salivary microbiota.³⁴ *Streptococcus* were the most numerous of the bacterial groups assayed since all samples showed average counts above 8 log copy numbers/mL. Average counts for *Lactobacillus* and *Prevotella* were about 7 log copy numbers/mL (Table 2). On the other hand, among all the bacterial groups analyzed, the lowest counts were recorded for *Actinomyces* that did not reach values higher than 5 log copy numbers/mL, which in turn was the average value recorded for *Fusobacterium*,

Haemophilus, *Neisseria* and *Veillonella*. The results are in agreement with metagenomic studies which reveal that the oral cavity is generally dominated by *Streptococcus* species.^{8,21}

The Student *t*-test for dependent samples showed no significant differences between samples before and after the 4-week study period, with *P* values higher than 0.1 in all cases (Table 2), indicating that no differences in bacterial counts could be attributable to the red wine intake. Due to the high variations in counts between individuals, *t*-test analyses were also performed distinguishing subjects grouped by initial counts being either higher or lower than the average values. Again, the results indicated no statistical differences in counts of any of the bacterial groups assayed.

The stability of numbers and composition of the saliva microbiota over the 4-week period of red wine intake shown in this work is in agreement with metagenomic studies indicating high levels of variability of human-associated microbial communities among individuals and minimal variations over time within a given human body habitat, such as the oral cavity.⁸ This oral stability is kept despite age, gender or daily ambient fluctuations including diet, such as the similar salivary microbiota observed in individuals with omnivore, ovo-lacto-vegetarian or vegan dietary habits.¹² Longitudinal surveys of salivary bacterial communities in twins from adolescence to adulthood have shown a long term stable microbiome in the saliva, more related to cohabitation of individuals than weight class or gender.²³ In this regard, one element to be considered for stable oral microbiota is the short time of exposition of this community to polyphenols in comparison with the estimated accumulation of dietary polyphenols in colon.³⁵ Accordingly, a recent study with 12 individuals showed that salivary antioxidant capacity did not increase after drinking 125 mL of red wine.¹⁹ Another relevant consideration is resilience of the oral microbiota, which implies the degree to which the microbial community returns to its former state after a disturbance caused for example by a therapeutic intervention.³⁶

In conclusion, the results showed that daily consumption of 250 mL red wine during 4 weeks does not change the overall diversity and stability of representative bacterial groups of the human saliva. Microbial balance and homeostasis are maintained over time in the oral cavity and no major perturbation can be observed due to regular-moderate red wine consumption.

Conflict of Interest:

The authors declare that they have no conflict of interest.

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Legend to figure.

Fig. 1. PCR-DGGE band profiles from eight individuals before (b) and after (a) red wine intake obtained from saliva DNA and specific primers for *Bifidobacterium* (A) and *Streptococcus* (B). Bands 1: *Bifidobacterium dentium*, 2: *Bifidobacterium* spp., 3: *Alloscardovia omnicolens*, S: group *Streptococcus mitis*-*Streptococcus oralis*.

^a*P* values evaluated with the Student *t*-test for dependent samples

Table 1. Primer sets used in this study for PCR-DGGE and quantitative PCR.

Target group	Primer sequence 5'-3'	Annealing (°C)	Gradient (%)	Reference
PCR-DGGE				
Total bacteria	F: AACGCGAAGAACCTTAC+GC ^a R: CGGTGTGTACAAGACCC	56	30-60	[37]
<i>Lactobacillus</i>	F: GGAAACAGGTGCTAATACCG R: ATCGTATTACCGCGGCTGCTGGCA C+GC ^a	56	30-50	[27]
	F: GTTTGATCCTGGCTCAG R: CACCGCTACACATGGAG	66	nested	[27]
<i>Bifidobacterium</i>	F: GGGTGGTAATGCCGGATG R: GCCACCGTTACACCGGGAA+GC ^a	62	40-55	[28]

	F: CGGGTGCTICCCACTTTCATG	57	nested	[28]
	R: GATTCTGGCTCAGGATGAACG			
<i>Streptococcus</i>	F: AGATGGACCTGCGTTGT+GC ^a	55	25-50	[38]
	R: GTGAACTTTCCACTCTCACAC			
<i>Veillonella</i>	F: A(C/T)CAACCTGCCCTTCAGA	62	40-70	[39]
	R: CGTCCCGATTAACAGAGCTT+GC ^a			
<i>Prevotella-</i> <i>Porphyromon</i> <i>as-</i> <i>Bacterioides</i>	F: GGTGTCGGCTTAAGTGCCAT+GC ^a	68	40-58	[39]
	R: CGGA(C/T)GTAAGGGCCGTGC			
<i>Neisseria</i>	F: CTGGCGCGGTATGGTCGGTT	55	30-70	[40]
	R: GCCGACGTTGGAAGTGGTAAAG+ GC ^a			

Quantitative PCR		
Total bacteria	F: CGGTGAATACGTTC(C/T)CGG R: CGGTGTGTACAAGACCC	59 [41]
<i>Actinomyces</i>	F: CTCCTACGGGAGGCAGCAG R: CACCCACAAACGAGGCAG	60 [42]
<i>Bifidobacterium</i>	F: CTCCTGGAAACGGGTGG R: GGTGTTCTTCCCGATATCTACA	55 [43]
<i>Fusobacterium</i>	F: C(A/T)AACGCGATAAGTAATC R: TGGTAACATACGA(A/T)AGGG	54 [39]
<i>Haemophilus</i>	F: GGAGTGGGTTGTACCAGAAGTA GAT R: AGGAGGTGATCCAACCGC	55 [44]

<i>Lactobacillus</i>	F: TGGAAACAG(A/G)TGCTAATACC G	62	[41]
	R: GTCCATTGTGGAAGATTCCC		
<i>Neisseria</i>	F: CTGGCGCGGTATGGTCGGTT	55	[40]
	R: GCCGACGTTGGAAGTGGTAAAG		
<i>Prevotella</i>	F: CAC(A/G)GTAAACGATGGATGCC	55	[43]
	R: GGTCGGGTTGCAGACC		
<i>Streptococcus</i>	F: AGATGGACCTGCGTTGT	55	[38]
	R: GTGAACTTTCCACTCTCACAC		
<i>Veillonella</i>	F: A(C/T)CAACCTGCCCTTCAGA	62	[39]
	R: CGTCCCGATTAACAGAGCTT		

^aGC clamp at 5': CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCC

Table 2. Quantitative PCR counts (mean $\pm$ SD of copy number/mL and range values in brackets) for the microbial groups analyzed in saliva from individuals before and after the wine intervention study.

Bacteria	Control group (n=8)			Intervention group (n=14)		
	before	after	P^a	before	after	P^a
Total bacteria	8.04 $\pm$ 0.56 (7.05-8.57)	7.78 $\pm$ 0.38 (7.44-8.56)	0.176	7.98 $\pm$ 0.40 (7.47-8.60)	7.80 $\pm$ 0.61 (6.65-8.67)	0.133
<i>Actinomyces</i>	4.67 $\pm$ 0.52 (3.94-5.29)	4.46 $\pm$ 0.39 (4.07-5.13)	0.161	4.89 $\pm$ 0.32 (4.43-5.56)	4.77 $\pm$ 0.52 (3.88-5.63)	0.279
<i>Bifidobacterium</i>	6.52 $\pm$ 0.19 (6.38-6.90)	6.31 $\pm$ 0.19 (6.11-6.65)	0.091	7.01 $\pm$ 0.44 (6.22-7.60)	6.76 $\pm$ 0.45 (6.12-7.57)	0.131
<i>Fusobacterium</i>	5.41 $\pm$ 0.89 (4.14-6.93)	5.13 $\pm$ 0.57 (4.18-5.85)	0.327	5.52 $\pm$ 0.77 (4.37-6.89)	5.60 $\pm$ 0.92 (3.98-6.97)	0.600
<i>Haemophilus</i>	5.82 $\pm$ 0.81 (4.77-7.33)	5.61 $\pm$ 0.55 (4.87-6.38)	0.262	6.09 $\pm$ 0.71 (5.16-7.15)	6.23 $\pm$ 0.81 (4.86-7.54)	0.463
<i>Lactobacillus</i>	8.03 $\pm$ 0.52 (7.12-8.58)	7.70 $\pm$ 0.35 (7.34-8.38)	0.116	7.97 $\pm$ 0.37 (7.39-8.52)	7.77 $\pm$ 0.62 (6.58-8.55)	0.116
<i>Neisseria</i>	5.57 $\pm$ 1.08 (4.35-7.58)	5.30 $\pm$ 0.79 (4.43-6.70)	0.123	5.85 $\pm$ 0.86 (4.89-7.41)	5.93 $\pm$ 1.08 (4.10-7.64)	0.675
<i>Prevotella</i>	7.34 $\pm$ 0.89	7.40 $\pm$ 0.54	0.674	7.55 $\pm$ 0.39	7.48 $\pm$ 0.78	0.807

	(5.58-8.49)	(6.76-8.13)		(7.10-8.24)	(5.76-8.58)	
<i>Streptococcus</i>	8.32 ± 0.39	8.21 ± 0.41	0.398	8.52 ± 0.51	8.45 ± 0.63	0.382
	(7.76-8.74)	(7.64-8.97)		(7.56-9.13)	(7.04-9.10)	
<i>Veillonella</i>	5.57 ± 0.66	5.23 ± 0.31	0.123	5.27 ± 0.61	5.38 ± 0.64	0.552
	(4.35-6.18)	(4.70-5.62)		(4.55-6.24)	(3.97-6.25)	