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RESEARCH ARTICLE

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Cross-sectional comparative shotgun metagenomic analysis of the subgingival resistome in healthy subjects and patients with periodontitis from four countries

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ABSTRACT

Background: The oral cavity is a known reservoir of antibiotic resistance genes (ARGs), but little is known about their subgingival distribution across health states and regions.

Objective: This study aimed to characterize and compare the subgingival resistome and mobile genetic elements (MGEs) in healthy subjects (HS) and periodontitis patients (PP) from Belgium, Chile, Peru and Spain.

Design: Subgingival samples pooled from the deepest site of each quadrant of 40 HS and 40 PP were analyzed via shotgun metagenomic sequencing. After human DNA depletion, the microbial composition was assessed with MetaPhlAn 4.0; ARGs were identified using MEGAHIT and AMRFinderPlus; and MGEs with MGEfinder.

Results: ARG richness was significantly higher in PP (mean 3.98) than in HS (2.15). PP from Peru showed more ARGs than HS from Chile and Spain. In total, 28 ARGs were found, conferring resistance to eight antibiotic classes. β -lactam, tetracycline and aminoglycoside resistance were more abundant in PP. Macrolide resistance was lower in Chilean samples than in Peruvian and Spanish ones. Additionally, 99 MGE-associated genes were detected, with 16 differing by diagnosis and 78 by country.

Conclusions: Subgingival resistome profiles vary significantly by periodontal status and geography, underscoring the influence of clinical and regional factors on antimicrobial resistance in the oral microbiome.

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Introduction

Antibiotic resistance genes (ARGs) encode proteins that confer resistance through several mechanisms, including the modification or efflux of an antibiotic, or the alteration of its molecular target, thus hindering or impeding the interaction between the antibiotic and its target. The oral cavity is increasingly recognized as a significant reservoir of ARGs [1]. The numerous colonizable niches, the close proximity of species within biofilms and the high bacterial diversity of this environment facilitate gene coexistence and enhance transferability via mobile genetic elements (MGEs) [2]. Despite this, little is known about the distribution of ARGs in the oral microbiome. Many studies have analyzed the presence of ARGs in isolates obtained from different niches [3–5], in order to associate specific genes or families of genes with bacterial species or genera. However, this has limited conclusions to either culturable bacteria or species showing phenotypic antibiotic resistance. Metagenomic analyses overcome these constraints, revealing novel ARGs [6–8], but their application to subgingival microbiota is still limited [9,10], and further research is needed to evaluate and preserve their effectiveness in the treatments where they are applied.

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Periodontitis is an inflammatory disease whose primary etiology is the subgingival biofilm [11]. The virulence factors of biofilm bacteria trigger the host's immune response, which can destroy the alveolar bone that serves as a structural anchor for teeth, potentially leading to tooth loss. Antibiotics have been proposed as adjuvants of non-surgical periodontitis treatment, improving clinical outcomes when used [12]. Recent work suggests that antimicrobial resistance in subgingival bacteria can be detected before antibiotic treatments and remains similar after it [13]. However, in the advent of the post-antibiotic era, the use of antibiotics should be strictly monitored and guided by empirical data to prevent misuse and the accelerated spread of antibiotic resistance [14].

Moreover, data on ARG distribution are heavily biased toward a small number of countries, most of which are among the wealthiest in the world, where policies regarding antibiotic administration tend to be strict and enforced by health administrations [15]. Furthermore, a recent review has shown different phenotypic resistance profiles in oral bacteria isolated from countries from Latin America, compared to countries from other continents [16]. Likewise, an even more recent study reported differences in the oral resistome analyzed from the saliva of Norwegian and Thai subjects [17]. Since antibiotic resistance is a global issue in which all states play a significant role, worldwide monitoring is essential to obtain the most reliable information for policy makers and stake holders [18].

Therefore, the objective of this study was to detect and analyze ARGs and MGEs in the subgingival metagenome of healthy subjects (HS) and patients with periodontitis (PP) across four countries—Belgium, Spain, Chile and Peru—with the latter two having limited prior research in this area.

Materials and methods

Study population

The study subjects included HS and PP attending university dental clinics in 4 different countries: Belgium (Katholieke Universiteit Leuven), Chile (Pontificia Universidad Católica de Chile), Peru (Universidad Científica del Sur) and Spain (Universitat Internacional de Catalunya) between 2017 and 2019. All four centers shared the same research protocol, which complied with the principles of the Declaration of Helsinki and was approved by their Ethics Committees (study numbers: S60696 [Belgium], 180111004 [Chile], 002-DACE-DAFCS [Peru] and ODO-2014-01 [Spain]). HS were considered free of gingivitis and other systemic diseases, with no history of periodontitis. PP had at least 18 natural teeth and generalized periodontitis of stages II, III or IV and grades B or C, and were systemically healthy. The exclusion criteria for both groups included having a history of previous periodontal treatment, smoking more than 10 cigarettes per day, pregnancy, breastfeeding, intake of antimicrobials or use of antiseptics in the preceding 6 months, intake of anti-inflammatory medicines in the preceding 4 months and recurrent use of antiseptics. From all the collected samples, a total of 80 were randomly selected (10 HS and 10 PP per country) and analyzed, in accordance with previous metagenomic resistome studies [19].

Sample collection

Subgingival samples were taken according to previously published methods [20]. Sampling was standardized across sites, with pooled paper-point samples from the deepest pocket per quadrant. Bleeding on probing (BOP), the clinical attachment level (CAL) and the periodontal probing depth (PPD) were measured in each sampling site. The probing depth and gingival recession/overgrowth (with the cemento-enamel junction as a reference point) were measured to the nearest 1 mm (buccally and orally of each root, and at each approximal site, both buccally and orally) using a periodontal probe. CAL was calculated using the sum of PPD and recession. BOP was evaluated 20 s after measuring PPD. Sampling areas were isolated with cotton rolls, and the supragingival plaque was removed using curettes. Then, two size-30 sterile paper points were inserted for 30 s in each site and pooled in a 2 mL tube that was frozen at -80°C and sent to the DentaId Research Center (Spain) without interrupting the cold chain.

DNA extraction and shotgun sequencing

DNA was extracted from the samples using the QiAamp DNA Mini Kit (Qiagen) following the manufacturer's instructions and quantified using a Nanodrop 2000C UV-vis spectrophotometer (Nanodrop

Technologies). A randomized subset of 80 DNA samples (10 of each diagnosis and country) was sent to MacroGen, Inc., to perform shotgun sequencing through Nextera XT DNA Library Preparation Kit and the NovaSeq6000 S4 Sequencing platform.

Data analysis

The quality of the reads was analyzed using FastQC [21] and trimmed, when necessary, using cutadapt [22]. Human DNA was depleted from samples using Kneaddata 0.12.1 pipeline [23] and the human genome reference GRCh38 was used as a reference [24]. The microbial composition of the samples was investigated to the species level using MetaPhlAn 4.0 [25]. The resulting frequency tables were analyzed using R packages ggplot2 [26] and stats [27] to graph and calculate differences between the microbiota grouped by the variables of the study. To identify antimicrobial resistance genes, contigs were created using MEGAHIT [28] and screened using NCBI's Antimicrobial Resistance Gene Finder (AMRFinderPlus) [29] default parameters (minimum identity and coverage of 90 and 50%, respectively). The presence of MGEs was inferred using MGEfinder [30] through the detection of genes associated with MGEs using the default parameters, which detect sequences of a minimum length of 70 base pairs. The relative abundance of ARGs, the antibiotic classes to which these ARGs may confer resistance, and MGEs were compared according to the subjects' country of origin and diagnosis using pairwise Wilcoxon rank sum tests with Benjamini-Hochberg-adjusted p -values [31]. The Spearman rank correlation coefficient was calculated to assess the correlation between the relative abundances of MGEs, bacterial species, antibiotic resistance, country of origin and diagnosis of the subjects. Relative abundances were centered-log ratio transformed [32] prior to correlation analysis and p -values were adjusted using the Benjamini-Hochberg correction.

Results

Eighty samples, equally distributed by diagnosis and country, were analyzed in this study. The clinical parameters of the subjects are displayed in Supplementary Table 1. A total of 876,717,640 reads were sequenced, averaging 10,958,970.5 per sample (standard deviation = 4,813,054.71). These reads clustered into 20,798,645 contigs, averaging 256,773.39 contigs per sample (SD = 132,465.47) and with a size in base pairs that ranged from 200 bp to 607,670 bp (mean = 479.54 bp, SD = 43.24 bp). This allowed the detection of 28 and 99 different ARGs and genes associated with MGEs, respectively. The number of ARGs was significantly higher in PP ($p = 0.005$), averaging 3.98 (SD = 3.13) different ARGs in PP and 2.15 (SD = 1.6) in HS. At the country level, no significant differences were observed. However, when combining country and diagnosis, PP from Peru (mean = 6.2, SD = 3.36) had significantly higher ARG richness, understood as the number of different ARGs, than HS from Chile (mean = 1.5, SD = 1.27, $q = 0.007$) and Spain (mean = 1.9, SD = 1.37, $q = 0.007$). Overall, the most detected ARG was *cfxA*, followed by *ermF* and *cata1*. On the other hand, the genes *tetB60*, *cfxA4*, *cfxA5*, *nimI*, *aph3IIIa*, *aph3la* and *blaCSP* were the least detected. Significant differences were observed regarding the distribution of ARGs, with certain genes, such as *ermF*, *cfxA* and *tetQ*, being more abundant in PP than in HS. Moreover, when samples were grouped by country and/or diagnosis, additional genes, such as *ermX*, *blaTEMcfxA4* or *tet37*, showed differential abundance (Supplementary Table 2). The detected ARGs had the potential to confer resistance to 8 classes of antibiotics: macrolides, β -lactams, tetracyclines, aminoglycosides, chloramphenicol, lincosamides, nitroimidazoles and sulfonamides (Supplementary Table 3).

The number of antibiotic classes per sample to which ARGs might confer resistance was significantly higher ($p = 0.001$) in PP, averaging 2.46 (SD = 1.43) and 1.51 (SD = 0.97) classes in PP and HS, respectively. Similarly, samples from Peru had a significantly higher ($q = 0.015$) average number of antibiotic classes associated with ARGs (mean = 2.55, SD = 1.28) than those from Chile (mean = 1.5, SD = 1.24) (Figure 1). Regarding potential resistance to antibiotic classes, significant differences between HS and PP were observed for β -lactams ($p < 0.001$), tetracyclines ($p = 0.006$) and aminoglycosides ($p = 0.048$), with PP showing higher resistance in all three classes. Moreover, when studying the country of origin of the samples, potential resistance to macrolides was significantly lower in Chilean samples compared to Peruvian and Spanish samples ($q = 0.005$ and $q = 0.007$, respectively) (Table 2).

Table 1. Prevalence (%) of antibiotic resistance genes in the samples, grouped by country of origin and diagnosis. ARG: antibiotic resistance genes, HS: healthy subjects, PP: patients with periodontitis.

Antibiotic class	ARG	All countries						Belgium			Chile			Peru			Spain		
		Total	HS	PP	Total	HS	PP	Total	HS	PP	Total	HS	PP	Total	HS	PP	Total	HS	PP
Aminoglycosides	<i>aph3IB</i>	3.75	0.00	7.50	5.00	0.00	10.00	0.00	0.00	0.00	0.00	0.00	10.00	0.00	0.00	0.00	0.00	0.00	0.00
	<i>aph6Id</i>	2.50	0.00	5.00	5.00	0.00	10.00	0.00	0.00	0.00	0.00	0.00	10.00	0.00	0.00	0.00	0.00	0.00	0.00
	<i>aph3Ia</i>	1.25	0.00	2.5	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	5.00	0.00	10.00	0.00	0.00	0.00
	<i>aph3IIla</i>	1.25	0.00	2.5	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	5.00	0.00	10.00	0.00	0.00	0.00
β-lactams	<i>cfxA</i>	37.50	20.00	55.00	30.00	20.00	40.00	40.00	50.00	40.00	60.00	40.00	10.00	40.00	10.00	70.00	30.00	10.00	50.00
	<i>blaTEM181</i>	23.75	20.00	27.50	25.00	10.00	40.00	20.00	15.00	10.00	20.00	30.00	30.00	30.00	30.00	30.00	25.00	30.00	20.00
	<i>cfxA3</i>	12.50	7.50	17.50	10.00	0.00	20.00	20.00	10.00	10.00	10.00	10.00	15.00	10.00	20.00	15.00	10.00	20.00	20.00
	<i>blaTEM</i>	8.75	7.50	10.00	10.00	10.00	10.00	10.00	15.00	20.00	10.00	10.00	5.00	0.00	10.00	5.00	0.00	10.00	10.00
	<i>blaTEM1</i>	3.75	5.00	2.50	10.00	20.00	0.00	0.00	0.00	0.00	0.00	0.00	5.00	0.00	10.00	0.00	0.00	0.00	0.00
	<i>blaCSP</i>	1.25	0.00	2.50	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	5.00	0.00	10.00	0.00	0.00	0.00	0.00
	<i>cfxA4</i>	1.25	0.00	2.50	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	5.00	0.00	10.00	5.00	0.00	10.00	10.00
	<i>cfxA5</i>	1.25	0.00	2.50	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	5.00	0.00	10.00	5.00	0.00	10.00	10.00
Chloramphenicol	<i>catA1</i>	26.25	22.50	30.00	30.00	20.00	40.00	20.00	20.00	20.00	20.00	25.00	20.00	30.00	30.00	30.00	30.00	30.00	30.00
Lincosamides	<i>lncC</i>	2.50	2.50	2.50	5.00	0.00	10.00	0.00	0.00	0.00	0.00	5.00	10.00	0.00	0.00	0.00	0.00	0.00	0.00
	<i>ermF</i>	32.50	10.00	55.00	25.00	10.00	40.00	20.00	20.00	0.00	40.00	20.00	45.00	20.00	70.00	40.00	10.00	0.00	70.00
	<i>mefA</i>	20.00	25.00	15.00	20.00	30.00	10.00	0.00	0.00	0.00	0.00	0.00	30.00	30.00	30.00	30.00	40.00	20.00	20.00
	<i>msrD</i>	20.00	25.00	51.00	25.00	40.00	10.00	0.00	0.00	0.00	0.00	0.00	25.00	20.00	30.00	30.00	40.00	20.00	20.00
Macrolides	<i>ermB</i>	18.75	17.50	20.00	20.00	20.00	20.00	10.00	10.00	10.00	10.00	30.00	30.00	30.00	30.00	15.00	10.00	20.00	20.00
	<i>ermX</i>	2.50	5.00	0.00	5.00	10.00	0.00	0.00	0.00	0.00	0.00	5.00	10.00	0.00	0.00	0.00	0.00	0.00	0.00
	<i>nimI</i>	1.25	0.00	2.50	0.00	0.00	0.00	0.00	0.00	0.00	0.00	5.00	0.00	10.00	0.00	0.00	0.00	0.00	0.00
	<i>sul2</i>	3.75	0.00	7.50	0.00	0.00	0.00	0.00	0.00	0.00	0.00	15.00	0.00	30.00	0.00	30.00	0.00	0.00	0.00
Nitroimidazoles	<i>tetQ</i>	22.50	7.50	37.50	20.00	10.00	30.00	20.00	15.00	10.00	20.00	35.00	10.00	60.00	20.00	40.00	0.00	10.00	10.00
Sulfonamides	<i>tet37</i>	21.25	15.00	27.50	25.00	30.00	20.00	20.00	20.00	20.00	20.00	35.00	10.00	40.00	5.00	20.00	0.00	10.00	10.00
	<i>tet32</i>	15.00	0.00	30.00	20.00	0.00	40.00	0.00	0.00	0.00	0.00	20.00	0.00	40.00	20.00	40.00	0.00	40.00	40.00
	<i>tetM</i>	13.75	7.50	20.00	20.00	10.00	30.00	10.00	5.00	0.00	10.00	10.00	10.00	10.00	10.00	20.00	10.00	30.00	30.00
	<i>tetW</i>	6.25	10.00	2.50	5.00	10.00	0.00	0.00	0.00	0.00	0.00	20.00	30.00	10.00	0.00	0.00	0.00	10.00	10.00
Tetracyclines	<i>tetX2</i>	2.50	0.00	5.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	5.00	0.00	10.00	5.00	0.00	0.00	0.00	10.00
	<i>tetB60</i>	1.25	2.50	0.00	0.00	0.00	0.00	0.00	5.00	10.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

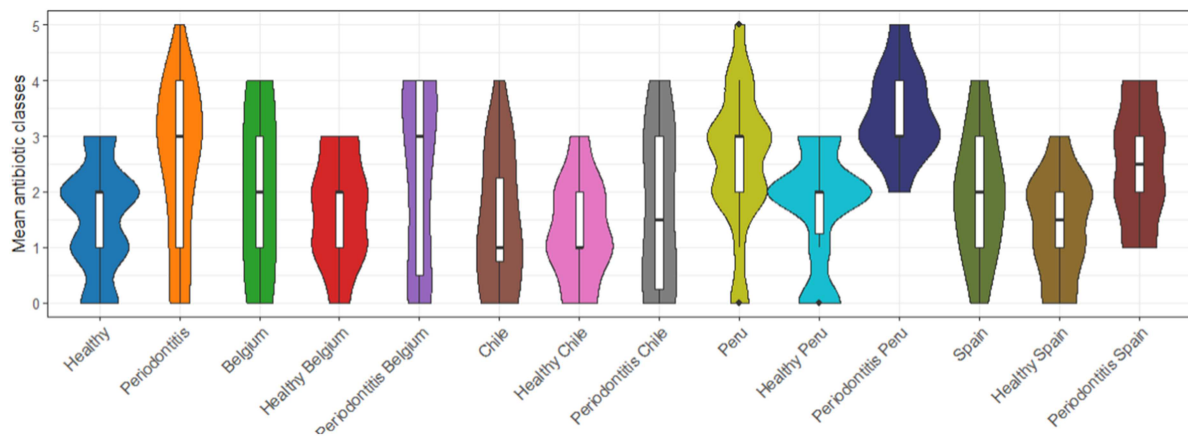


Figure 1. Mean number of different potential antibiotic resistance classes, as inferred from the antibiotic resistance genes detected.

MGEs were inferred in all samples, ranging from 1 to 24 different MGE-associated genes per sample (mean = 5.99, SD = 4.52), with the number of distinct MGEs differing significantly by country of origin and diagnosis. PP from Peru harbored significantly more distinct MGE-associated genes (mean = 12.3, SD = 5.96) than any other group, except for HS from Belgium, which had the second-highest diversity of these genes (mean = 9.11, SD = 4.04). On the other hand, HS from Spain was the group with the lowest diversity of MGE genes (mean 2.8, SD = 2.44). The same patterns were observed regarding the total number of MGE genes detected, with the addition of a significantly higher number of MGE genes in HS from Belgium than in HS from Chile and Peru. *ISSpn1* was the most detected MGE-associated gene in the samples, followed by *ISAeme22*, *ISPg6* and *ISBf8*, which are all transposases. Among these, *ISBf8* and *ISPg6* were significantly more abundant in PP than in HS, with no significant differences observed by country of origin. Differential abundance analysis revealed that up to 78 MGE genes varied significantly between countries, while up to 16 MGE genes varied significantly between diagnoses (Supplementary Table 4). Moreover, several conjugative transposons (CTs) or CT-associated genes were also detected, with up to 9 different CTs inferred from the samples (i.e. tn4400, tn4555, tn4351, tn6009, tn2009, tn5432, tn3-like, tn917 and CTnPi1). Of these, Tn4400 and Tn4555 were significantly more abundant in PP, with no differences between countries (data not shown).

A total of 364 bacterial species were identified, with a mean of 74.85 (SD = 30.57), 69.25 (SD = 32.11), 80.65 (SD = 56.20) and 47.65 (SD = 25.54) species in Belgian, Chilean, Peruvian and Spanish samples, respectively. With respect to diagnosis, an average of 50.97 (SD = 32.14) and 84.39 (SD = 38.93) species were detected in HS and PP, respectively. The relative abundances of the 15 most abundant microorganisms overall are displayed in Figure 2 and in Supplementary Figure 1.

Correlation analyses were conducted to examine the relationship between potential resistance to antibiotic classes, the relative abundance of the 10 most relatively abundant bacterial species, the 10 most relatively abundant genes associated with MGEs and subject's geographical origin and diagnosis. A statistically significant correlation was observed in several cases (Figure 3, Supplementary Tables 5–8). The highest Spearman correlation values were observed between *Streptococcus oralis* and *Streptococcus mitis* (coefficient = 0.73), *Porphyromonas gingivalis* and *Tannerella forsythia* (coefficient = 0.59), *Veillonella parvula* and lincosamides resistance (coefficient = 0.56), *Fusobacterium nucleatum* and chloramphenicol (coefficient = 0.56), and between *T. forsythia* and PP (coefficient = 0.53). A statistically significant correlation was also observed between macrolide resistance and the relative abundance of MGE-associated genes such as *ISSpn1* and *ISAeme22* (coefficients = 0.26 and −0.26, respectively), whereas tetracycline resistance was significantly correlated with *ISBf8* and *ISAeme22* (coefficients = 0.26 and −0.22, respectively). While *ISHyma1*, *ISSpn1* and *ISSpn4* showed a significant positive correlation with commensal bacteria such as *S. mitis*, *S. oralis* and/or *Rothia dentocariosa* (Supplementary Tables 5–8) and *ISBf8* showed a significant positive correlation with periodontopathogens such as *T. forsythia* (coefficient = 0.29) (Supplementary

Table 2. Prevalence (%) of potential resistance to antibiotic classes, inferred by the antibiotic resistance genes detected. Numbers in superscripts within the same antibiotic class indicate those groups with statistically significant differences. HS: healthy subjects, PP: patients with periodontitis.

		Aminoglycosides	β -Lactams	Chloramphenicol	Lincosamides	Macrolides	Nitroimidazole	Sulfonamides	Tetracyclines
Belgium	Total	5	60.00	30.00	5.00	50	0.00	0.00	50.00
	HS	0.00	40.00	20.00	0.00	50.00 ³	0.00	0.00	40.00
	PP	10	80.00	40.00	10.00	50.00	0.00	0.00	60.00
Chile	Total	0.00	70.00	20.00	0.00	25.00 ^{1,2}	0.00	0.00	35.00
	HS	0.00	70.00	20.00	0.00	10.00 ^{3,4}	0.00	0.00	30.00
	PP	0.00	70.00	20.00	0.00	40.00 ⁵	0.00	0.00	40.00 ²
Peru	Total	15.00	75.00	25.00	5.00	65.00 ¹	5.00	5.00	60.00
	HS	0.00	50.00 ^{3,4}	20.00	10.00	50.00 ⁴	0.00	0.00	40.00 ^{4,5}
	PP	30.00	100.00 ⁴	30.00	0.00	80.00 ⁵	10.00	10.00	80.00 ^{4,5}
Spain	Total	0.00	70.00	30.00	0.00	65.00 ²	0.00	0.00	30.00
	HS	0.00	50.00 ^{2,3}	30.00	0.00	50.00	0.00	0.00	10.00 ^{3,4}
	PP	0.00	90.00 ²	30.00	0.00	80.00	0.00	0.00	50.00 ³
All countries	Total	5.00	68.75	26.25	2.50	51.25	1.25	1.25	43.75
	HS	0.00 ¹	52.50 ¹	22.5	2.50	40	0.00	0.00	30.00 ¹
	PP	10.00 ¹	85.00 ¹	30.00	2.50	62.5	2.5	2.50	57.50 ¹

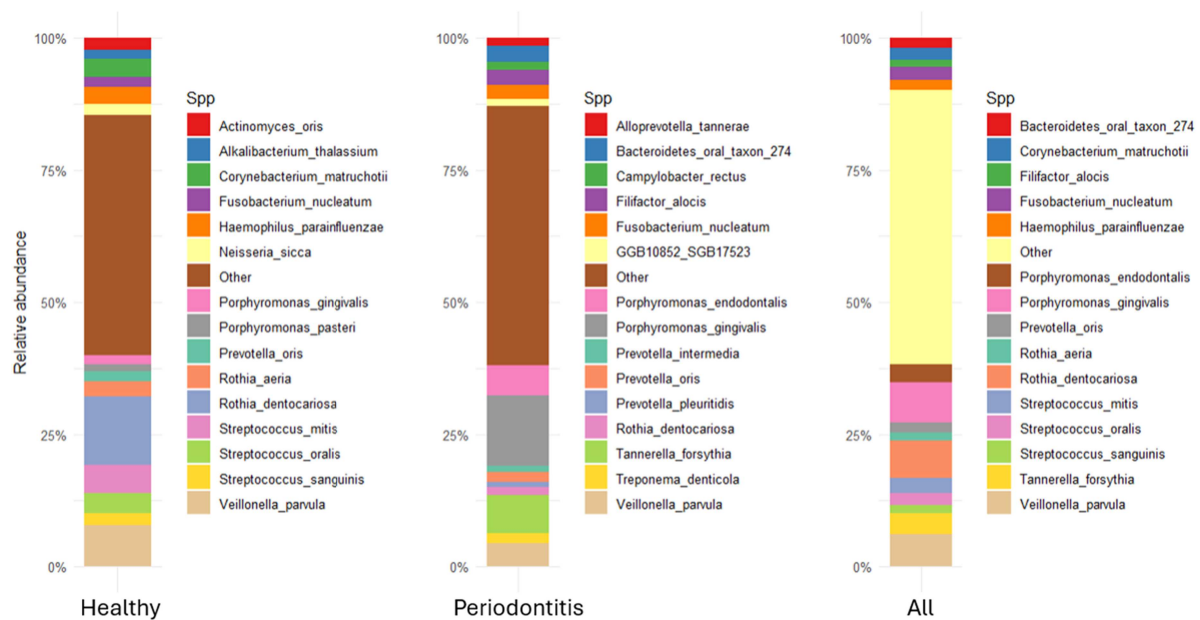


Figure 2. Relative abundance of the 15 most abundant bacterial species. The category 'Other' includes all remaining taxa detected in the samples. From left to right, healthy subjects, patients with periodontitis and all samples.

Table 2). Moreover, a significant negative correlation was observed between the relative abundance of potential resistance to β -lactams and to macrolides (coefficient = -0.35) and between chloramphenicol and tetracyclines (coefficient = -0.23). Conversely, a significant positive correlation between β -lactams and chloramphenicol resistance was detected (coefficient = 0.32) (Figure 3 and Supplementary Table 2). Similar to what was detected with hypothesis tests, Chilean samples showed a negative correlation with the relative abundance of potential resistance to macrolides (coefficient = -0.31) and a positive one with β -lactam resistance (coefficient = 0.28). Moreover, significant correlations were also observed between Spanish samples and macrolide resistance, and between Peruvian samples and aminoglycoside resistance (both coefficients = 0.24).

Discussion

Antibiotic resistance is a global issue that needs constant monitoring [18]. It is well known that when exposed to selective pressure over time, bacteria can overcome almost all antibiotics through adaptive mechanisms encoded in ARGs [33]. Different policies regarding antibiotic use create varying levels of selective pressure. Therefore, in the absence of a global, enforced regulation, it is reasonable to expect that regions with differing antibiotic practices will also show differences in the prevalence and abundance of ARGs [34]. This was observed in our study, where several ARGs showed differential relative abundances and correlation patterns depending on the country of origin of the samples.

In this study, we analyzed the resistome of the subgingival microbiota of 40 HS and 40 PP from four different countries: Belgium, Chile, Peru and Spain. Of these samples, 83.75% had at least one ARG, 68.75% had more than one and 25% had more than four different ARGs. Samples harbored an average of 3.09 ARGs (SD = 2.65), with a sample from a Peruvian PP containing 15 distinct ARGs. This supports the idea that the oral environment, particularly the subgingival niche, acts as a reservoir of ARGs, due to its high microbial diversity, close proximity of microorganisms and their embedding in a biofilm matrix that facilitates genetic exchange [2]. Putative resistance to 8 different antibiotic classes was detected, with β -lactams (68.75%), macrolides (51.25%) and tetracyclines (43.75%) being the most common. These classes are, or have been, the most widely used antibiotics for treating various human diseases, including oral diseases such as periodontitis or endodontitis [35,36], which may have exerted selective pressure favoring the conservation of resistance genes. However, although β -lactams and macrolides continue to be widely

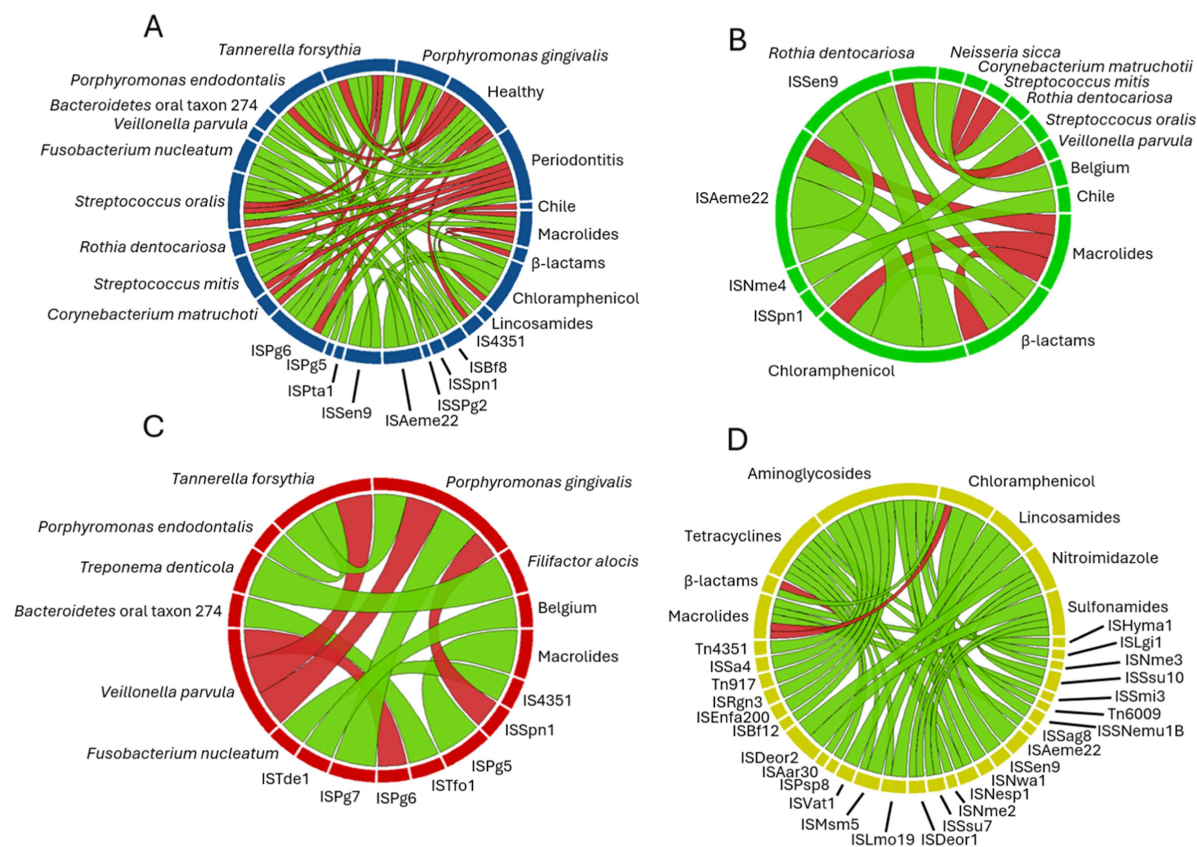


Figure 3. Correlation plots among genes associated with diagnosis, sample country of origin, mobile genetic elements (MGEs), bacterial species and potential antibiotic resistance classes. Positive correlations are shown in green, while negative correlations are shown in red. Only statistically significant correlations ($p < 0.05$) with absolute values greater than 0.3 are shown in the figures. In plots A–C, only the 10 most abundant MGE-associated genes were considered, while in plot D, all MGE-associated genes were considered. A) All samples. B) Considering only healthy subjects. C) Considering only patients with periodontitis. D) Comparing only resistance to antibiotic classes and all MGEs.

used in medicine, including in dental practice, this is not the case for tetracyclines, which are prescribed less often [37,38]. There might be multiple reasons for this, one being the co-selection of tetracycline resistance genes given their presence in mobile genetic elements carrying other genes under selective pressure [39,40], and another the extensive use of tetracyclines in husbandry, which far exceeds the consumption of β -lactams and macrolides in this context [41]. Mobile genetic elements such as the conjugative transposons of the family Tn916/1545 or Tn916-like usually carry tetracycline resistance genes in their sequences [42]. In this study, these transposons were detected and have been described as highly promiscuous, capable of propagating across diverse oral bacterial taxa [43] and disseminating their associated ARGs throughout whole bacterial communities. Even more, recent studies showed a non-statistically significant increase in the prevalence of tetracycline resistance genes in the oral microbiota following chlorhexidine treatments [44], reinforcing the idea of a possible co-selection mediated by MGEs carrying *tet* genes. On the other hand, tetracyclines have been extensively used in agriculture as questionable growth promoters [45,46]. This usage involves administering subtherapeutic doses of the antibiotic, which is hypothesized to increase nutrient absorption and gastrointestinal microbial regulation [47], aiming for greater, quicker and more profitable animal growth at the expense of spreading antimicrobial resistance and compromising global health [48,49]. Fortunately, this practice is increasingly regulated, with the European Union leading the way by implementing a ban in 2006 [50]. In the 2022 eighth annual round of data collection on antimicrobial agents intended for use in animals, 71.7% of the participating countries reported no use of antimicrobials as growth promoters, despite only 58.55% having legislation addressing this issue [51]. However, the situation in the Americas was the worst among those

studied, with 73.91% of the included countries still using antimicrobials as growth promoters. The two American countries included in this study have different policies regarding this issue. While Chile has banned this practice since 2017 [52], antibiotics are still commonly used in Peru as growth promoters [53], which could explain the greater richness of ARGs related to tetracycline resistance in the Peruvian PP of this study. Moreover, based on the detected genes, statistically significant differences in resistance to β -lactams, tetracyclines and aminoglycosides were observed between HS and PP. A higher number of β -lactam resistance-associated genes was to be expected in PP, given that amoxicillin is one of the most prescribed antibiotics, also in oral treatments [36,54]; thus, selective pressure would be expected to increase their abundance. However, aminoglycosides are not a usual drug of choice in the treatment of periodontitis, so their association with PP might be surprising at first glance. Nevertheless, it has been previously suggested that aminoglycoside resistance genes are usually found together with β -lactam resistance genes [55]. Therefore, it is plausible that the same mobile genetic elements that are carrying the β -lactam resistance genes are also carrying the aminoglycoside resistance genes and being kept together under co-selective pressure. In addition, a similar pattern in the most detected genes in PP was observed when comparing to a recent study performed in Germany [56], in which *tetQ*, *cfxA*, *tet32*, *mefA* and *msrD* showed the highest prevalence in subgingival samples of patients with periodontitis. This might suggest that the microbial profile associated with the periodontal diagnosis may exert a stronger influence on the dominant ARGs of the subgingival resistome than the country of origin of the samples.

Although regulations and policies differ among the four study countries – two from the European Union and two from South America – differences in potential antibiotic resistance were observed only for macrolide resistance, with Chilean samples showing significantly lower potential resistance to macrolides than those from Spain and Peru. Previous reports have indicated that macrolide resistance in *Streptococcus pneumoniae* isolates from Chile is among the least prevalent in South America, along with Brazil, Colombia and Uruguay. On the other hand, Peruvian isolates showed a prevalence of between 24.8 and 78.8% for macrolide resistance [57]. Given that *S. pneumoniae* is a steady member of the oral microbiome, these results might serve as an indicator of the levels of macrolide resistance in oral bacteria and could explain the lower potential resistance to macrolide found in Chilean samples compared to Peruvian samples. Also, Chile is recognized for its stricter regulations on antibiotic consumption compared to other South American countries [58]. Consequently, a lower prevalence of macrolide resistance is to be expected. Although Spain must comply with strict European Union regulations, macrolide consumption remains relatively high in both humans and animals [14], which may help explain these results.

The MGE-associated genes detected in the highest amounts were *ISSpn1*, *ISAeme22*, *ISPg6* and *ISBf8*, with total relative abundances of 11.39, 6.91, 5.6 and 4.23%, respectively. These genes code for transposases that could be part of conjugative transposons. Such is the case of *ISBf8*, which is inserted in the *Tn4555* transposon from *Bacteroides fragilis* [59], which also carries the gene *cfxA*, a gene that confers resistance to cephalosporins [60]. *Tn4555* has previously been associated with bacteria of the genera *Prevotella* and *Capnocytophaga* [61], which are usually enriched in PP [62,63] and might also explain the significantly higher abundance of *cfxA* in the PP of this study. *ISAeme22*, *ISSpn1* and *ISPg6*, previously detected in *Aeromonas media*, *S. pneumoniae* and *P. gingivalis*, respectively [64], are not associated with MGEs that carry described ARGs in their vicinities (data not shown). Therefore, their selection has little to do with antibiotic usage, which could explain the lack of significant differences observed between the groups of this study. Other MGE-related genes that were highly associated with PP, despite their lower relative abundances, were *IS4351*, *ISTf01*, *ISCagr1*, *ISPg7* and *ISOrsp1*. These transposases have been detected in obligate anaerobes commonly associated with periodontitis, such as *P. gingivalis*, *T. forsythia* and *Campylobacter* sp. [64,65]. With the exception of *IS4351*, ARGs were not found in proximity to these transposases (data not shown), making it difficult to determine whether their increased relative abundance was driven by selective pressure or by the higher proportions of the microorganisms carrying them. Nevertheless, correlation analyses of MGE-associated genes and potential antibiotic resistance showed that most classes were significantly positively correlated with MGEs, potentially indicating that these elements have incorporated ARGs into their sequences and are now propagating them. However, more research on this topic is needed to confirm this hypothesis. Moreover, significant differences in the relative abundance of MGEs were also observed when comparing countries (Supplementary Table 4). These differences, along with the variability in ARGs and microbial profiles, underscore the importance of viewing bacterial

communities – and their derived elements – as modifiable entities influenced by the country of origin of the samples. This is of critical importance, as it suggests that strategies aiming to improve – or at the very least, not exacerbate – the current antibiotic resistance landscape should account for the variability associated with the country of origin.

This study is not without limitations. Although the sample size and sequencing depth were consistent with those used in other studies [19], a larger and more geographically and demographically diverse set of samples would strengthen the geographical findings, while deeper sequencing might ensure the identification of all ARGs and MGEs. Moreover, despite their numerous advantages, high-throughput sequencing metagenomic studies also have shortcomings – chiefly the need for robust databases to maximize analysis sensitivity and the limitation that only previously characterized genes can be detected. Nevertheless, progress is being made towards predicting ARGs based only on sequencing data [66], which will increase the scope of metagenomic studies in the near future. Also, multivariate adjustment, which provides a better explanation of the results, was not performed due to the low number of samples in the smallest group, which may result in insufficient statistical power to detect interactions between variables [67,68]. Finally, phenotypic validation of the potential resistance to antibiotic classes conferred by ARGs, despite the technical challenges involved, would be necessary to confirm the findings of this study and to assess the level of resistance provided by ARGs.

In conclusion, the results of the study show that the country of origin of the subjects and their periodontal diagnosis may have an impact on the oral resistome. This is particularly important given the rise in antibiotic resistance and the increasing need for tailored treatments, not only to ensure that patients are given antimicrobials that will be effective but also to limit the propagation of ARGs. Future studies will be needed to assess the phenotypical and clinical impact of these differences, as well as to incorporate data from the widest possible range of countries to broaden our understanding of the resistome and how to mitigate it.

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Author contributions

CRediT: **A. Arredondo:** Conceptualization, Data curation, Formal analysis, Software, Visualization, Writing – original draft, Writing – review & editing; **G. Álvarez:** Investigation, Writing – review & editing; **S. Isabal:** Investigation; **W. Teughels:** Investigation, Resources, Writing – review & editing; **I. Laleman:** Investigation, Resources, Writing – review & editing; **M. J. Contreras:** Investigation, Resources, Writing – review & editing; **L. Isbej:** Investigation, Resources, Writing – review & editing; **E. Huapaya:** Investigation, Resources, Writing – review & editing; **G. Mendoza:** Investigation, Resources, Writing – review & editing; **C. Mor:** Investigation, Resources, Writing – review & editing; **J Nart:** Investigation, Resources, Writing – review & editing; **V. Blanc:** Resources, Supervision, Writing – review & editing; **R. León:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing.

Disclosure statement

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Data availability statement

The raw sequence data of this study have been uploaded to the BioProject repository (PRJNA1183294) of the National Center for Biotechnology Information.

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