Comparative 16S rRNA gene sequencing study of subgingival microbiota of healthy subjects and patients with periodontitis from four different countries

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Abstract

Aim: To investigate the differences between the subgingival microbiota of healthy subjects and that of periodontitis patients from four different countries through a metagenomic approach.

Materials and methods: Subgingival samples were obtained from subjects from 4 different countries. Microbial composition was analysed through high-throughput sequencing of the V3-V4 region of the 16S rRNA gene. The country of origin, the diagnosis, and the clinical and demographical variables of the subjects were used to analyse the microbial profiles.

Results: In total, 506 subgingival samples were analysed - 196 from healthy subjects and 310 from patients with periodontitis. Differences in richness, diversity and microbial composition were observed when comparing samples pertaining to different countries of origin and different subject diagnoses. Clinical variables, such as bleeding on probing, did not significantly affect the bacterial composition of the samples. A highly conserved core of microbiota associated with periodontitis was detected, while the microbiota associated with periodontally healthy subjects was much more diverse.

Conclusions: The periodontal diagnosis of the subjects was the main variable explaining the composition of the microbiota in the subgingival niche. Nevertheless, the country of origin also

had a significant impact on the microbiota and is therefore an important factor to consider when describing subgingival bacterial communities.

Clinical Relevance

Scientific rationale for the study: Subgingival microbiota is usually described based on samples from restricted populations. However, geographical and cultural differences might have an impact on the composition of these bacterial communities.

Principal findings: Significant differences in the alpha and beta diversity of subgingival microbiota were observed when comparing samples by the country of origin. However, subject periodontal diagnosis had a greater impact.

Practical implications: Due to the several differences observed in the microbial composition according to both the subject's country and the diagnosis, it might be important to acknowledge this variable when associating subgingival microbiota with either a status of health or of periodontitis.

Keywords: microbiome, periodontitis, subgingival, high-throughput sequencing

1. Introduction

Periodontitis is a chronic inflammatory disease of the periodontal tissues, which can lead to tooth loss due to the destruction of the alveolar bone that holds the teeth. This inflammatory state has been associated with subgingival dysbiotic biofilm[1]. Many efforts have been made to determine the bacteria involved in periodontitis. What started with the identification of only culturable bacteria[2,3], evolved to the molecular detection of the whole subgingival microbiome thanks to high-throughput sequencing (HTS) technologies[4,5]. However, when these technologies appeared, their cost was, and still is, too expensive for some research teams, restricting its usage and preparing the ground for bias due to the overwhelming amount of data from economically richer countries. Moreover, those studies that included HTS in their analyses presented results that are difficult to collate due to methodological differences. Different methods including sample collection, preparation of libraries and even bioinformatic assessment of sequences can significantly alter the output of the analysis, which hinders replication of the studies[6–8]. In addition, potential causes of bias, such as the clinical conditions of the subjects included in the study, their habits, ethnicity, and low numbers of samples, may influence the outcome of the studies and therefore the comparison of the results [2,9–11]. Despite these issues, microbial profiles

obtained through HTS are usually discussed without considering their geographical origin, but rather relying more on the clinical variables of the subjects from whom they were obtained.

Healthy microbiomes have been described to exhibit a high level of interindividual variability[12]. Moreover, previous analyses have detected significant differences when studying the salivary microbiomes of healthy subjects from different geographical locations and of different ethnicities[10]. On the other hand, the microbiome associated with periodontitis is dominated by certain species that introduce metabolic functions and virulence factors that are related with the onset or the progression of the disease[13]. Despite the specialised core of periodontitis-associated bacteria, some studies have found significant differences between the relative abundance and prevalence of such bacteria when comparing subjects of different ethnicities or geographical locations[14–16].

Given that the determination of bacterial profiles can be key to designing future periodontal treatments[17], it seems essential to acknowledge the particular differences that might exist between the microbiota of different geographical origins.

The aim of this study was to perform a metagenomic comparative analysis of the subgingival microbiota of healthy subjects and patients with periodontitis from four different countries, following the same methodological approach to thereby reduce potential biases.

2. Material and methods

2.1. Study population

This was a case control study, including periodontally healthy subjects (HS) and patients with periodontitis (PP) from 4 different countries: Belgium, Chile, Peru and Spain. Subjects were volunteers attending the dental clinics of the Faculties of Dentistry of Katholieke Universiteit Leuven (Belgium), Pontificia Universidad Católica de Chile (Chile), Universidad Científica del Sur (Perú) and Universitat Internacional de Catalunya (Spain) between 2017 and 2019. The dental clinics recruited at least 30 subjects of each group (healthy and periodontitis). Each centre had the same research protocol, which complied with the principles of the Declaration of Helsinki and was approved by their Ethics Committee (study numbers: S60696 [Belgium], 180111004 [Chile], 002-DACE-DAFCS [Peru], and ODO-2014-01 [Spain]). Inclusion criteria for healthy subjects included being free of gingivitis and systemic disease, and not having a history of periodontitis. The inclusion criteria for patients with periodontitis included being systemically healthy, retaining at least 18 natural teeth and having a stage II, III or IV and grade B or C

generalised periodontitis according to the latest classification[1]. Exclusion criteria for both groups included having a record of previous periodontal treatment, smoking more than 10 cigarettes per day, pregnancy, breastfeeding, intake of antimicrobials or use of antiseptics during the previous six months, and intake of anti-inflammatory medicines in the previous four months.

2.2. Sample collection

Subgingival samples were taken from the deepest periodontal pocket of each quadrant, gathering clinical parameters (bleeding on probing [BOP], clinical attachment level [CAL] and periodontal probing depth [PPD]) of each sampled site. The probing depth and gingival recession/overgrowth (with the cementoenamel junction [CEJ] 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 the PPD and the recession. BOP was evaluated 20 s after probing the depth of the pocket; the scores were 0 (absent) and 1 (present). Each area was isolated with cotton rolls and the supragingival plaque was removed using curettes. Then, two size 30 sterile paper points were inserted for 30 seconds in each periodontal pocket and pooled in a 2ml screw cap microcentrifuge tube that was frozen at -80°C and sent to the DENTAID Research Center (Spain) without interrupting the cold chain.

2.3. DNA extraction, 16S rRNA gene amplification and sequencing

DNA was extracted from the samples using the extraction QiAamp DNA Mini kit (Qiagen) following the manufacturer's instructions and quantified using a Nanodrop 2000C UV-vis spectrophotometer (Nanodrop Technologies). The 16S V3-V4 regions were amplified through 25 cycles of PCR using the primers forward 5'–TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG -3' and reverse 5'–GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC -3', which included the overhang adapters for the indexes in their 5' region. The following PCR clean-up was performed using AMPure XP beads (Beckman Coulter) following the manufacturer's instructions. Then, indexes (Illumina Inc.) were added with a second 8-cycle PCR which was purified again using the AMPure XP beads. Indexed sequences were quantified using the Qubit 1X dsDNA HS Assay Kit in a Qubit 4 fluorometer (Thermo Fisher Scientific) and pooled at a final equimolar concentration of 4 nM. Sequencing was performed using a MiSeq Reagent Kit V3 600-cycle Kit (Illumina Inc.), loading the pooled samples in the sequencer at a final concentration of 11 pM and occupying 20% of the flow cell with the PhiX control (Illumina Inc.), as recommended by the manufacturer's instructions.

2.4. Data analysis

Differences among the clinical and demographic (age and gender) characteristics of the volunteers were analysed using linear models and either the chi square or the Fisher's exact tests. Sequences were analysed using the DADA2 1.18 pipeline[18] to cluster them into amplicon sequence variants (ASVs), and the SILVADB[19] database to classify each ASV at the genus level. Alpha diversity was analysed with the R package Phyloseq 1.42[20], using the Chao1 and Shannon indexes as estimators of richness and diversity, respectively. Statistical differences of these estimators based on the variables of the study were assessed using the Kruskal-Wallis test and the post hoc Dunn's test of the package's stats 4.1.2[21] and FSA 0.9.1[22], respectively. For beta diversity, Principal Coordinates Analysis (PCoA) of weighted UniFrac distances was conducted with the Phyloseq package, and differences among the defined groups were assessed using the PERMANOVA test implemented in the adonis function of the vegan 2.5-7 package[23]. Differential abundance of the identified taxa was studied using the R packages Phyloseq and DESeq2 1.30.1[24], filtering the differences by a log2 Fold Change (L2FC) absolute value higher than 2 and by their base mean value, not considering ASVs with a base mean value pertaining to the lowest quartile. The association between the patterns of bacterial genera and the combination of the variables of the study was assessed using the R package indicspecies 1.7.9[25]. Graphical models of the relationship of the microorganisms, based on their relative abundance, were made with the SPIEC-EASI 1.1.1 (sparse inverse covariance estimation for ecological association inference)[26] package and Gephi 0.9.2[27] following the pipeline described by Dennis et al[28].

3. Results

3.1. Subjects' characteristics and clinical periodontal status

This study included 506 volunteers attending dental clinics located in 4 different countries: Belgium (38 HS and 38 PP), Chile (38 HS and 43 PP), Peru (64 HS and 108 PP) and Spain (56 HS and 121 PP). Table 1 describes the clinical and demographic characteristics of the subjects included in the study. Significant differences ($p < 0.05$) between some of the groups (especially between HS and PP) were found for most of the study variables analysed.

3.2. Microbiome analysis

The analysis of the bacterial diversity and richness of the different populations showed significant differences according to the Chao1 and Shannon indexes (Figures 1 and S1, Table S1). A higher richness and diversity were observed in PP compared to HS ($p = 5.48 \times 10^{-6}$, $p = 1.1$ x

10^{-6}), without considering their country of origin. Peruvian samples also showed more bacterial richness and diversity than Belgian (p = 1.8 x 10^{-7}, p = 1.0 x 10^{-4}) and Spanish (p = 4.9 x 10^{-6}, p = 1.0 x 10^{-4}) samples, and a higher richness than Chilean (p = 5.5 x 10^{-6}) samples. Moreover, Peruvian HS (p = 1.1 x 10^{-2}) and Spanish HS (p = 1.6 x 10^{-2}) showed a higher richness than Belgian HS. When comparing periodontitis groups, both richness and diversity were higher in Peruvian samples than in Belgian (p = 1.3 x 10^{-5}, p = 2.0 x 10^{-4}), Chilean (p = 4.0 x 10^{-4}, p = 1.5 x 10^{-2}) and Spanish (p = 5.7 x 10^{-9}, p = 6.5 x 10^{-8}) samples.

The distribution of the microbial composition was assessed using PCoA plots of weighted UniFrac. PCoA ordination analysis showed significant differences in microbial composition when comparing diagnosis and country of origin. However, diagnosis (Figure 2A) had a higher contribution (adonis p value = 0.001, R^2 = 0.16) than country of origin (adonis p value = 0.001, R^2 = 0.03; Figure 2B). Similarly, the microbiota of the HS group showed differences according to the country of origin (HS: adonis p value = 0.001, R^2 = 0.09). When analysing each country individually, diagnosis explained more microbial variability in Belgium (adonis p value = 0.001, R^2 = 0.42) than in the other countries, Peru being the least explained by this variable (adonis p value = 0.001, R^2 = 0.09) while Spain (adonis p value = 0.001, R^2 = 0.19) and Chile (adonis p value = 0.001, R^2 = 0.26) were in-between (Figure S2). BOP, CAL, PPD, age and gender were not able, on their own, to significantly determine the microbial composition of the studied populations (Table S2 and Figure S2).

Of the more than 500 bacterial genera detected in the samples, the 10 genera with more relative abundance were *Fusobacterium, Streptococcus, Prevotella, Veillonella, Leptotrichia, Porphyromonas, Neisseria, Treponema, Rothia* and *Fretibacterium* (Figure 3), accounting for slightly more than 50% of the sequences detected in every country and diagnosis. The main differences in the distribution of these genera were due to the periodontal status of the subjects, regardless of their country of origin.

Significant differences were observed when analysing the differential abundance of the bacterial genera detected in the samples using the DESeq2 package (Figure 4, Table S3). For instance, genera that contain known periodontopathogens, such as *Porphyromonas gingivalis, Filifactor alocis, Tannerella forsythia* and *Treponema denticola,* showed a significantly higher relative abundance in PP from most of the countries. Moreover, other genera, such as *Acholeplasma, Bacteroides, Desulfobulbus, Desulfovibrio, Filifactor, Oceanivirga*, *Odoribacter* and *Phocaeicola*, were significantly more relatively abundant in PP from the 4 countries (Figure 4). On the other

193 hand, no bacterial genus was significantly more abundant in the HS samples from all the
194 countries.

195 However, some genera, such as *Cutibacterium*, *Defluviitalea*, *Enterococcus*, *Exiguobacterium*,
196 *Haemophilus*, *Rothia* and *Sphingobium*, were significantly more abundant in HS from almost all
197 countries. Differences between countries, regardless of the health status, were also observed
198 (Figure S4). For instance, the genus *Xanthomonas* was significantly more abundant in Belgian
199 samples than in samples from the rest of the countries, while the opposite happened with the
200 genera *Oceanivirga, Lactobacillus* and *Acinetobacter*. Similarly, the genus *Staphylococcus* was
201 more abundant in Peruvian samples than in those of the other countries. Further genera showed
202 a more diverse pattern of differential abundance, such as *Paracoccus,* which was significantly
203 more abundant in Spanish and Peruvian samples than in Belgian and Chilean samples.

204 Microbial signatures of each country and diagnosis were determined using the R package
205 indicspecies. The 5 bacterial genera more significantly ($p < 0.05$) associated with country of
206 origin and diagnosis are shown in Table 2. The whole table, with the complete microbial
207 signatures, is displayed in the supplementary material (Table S4).

208 Network plots, indicating the influence on relative abundance between genera, were made for
209 each subject diagnosis and country of origin (Figure 5) using the SPIEC-EASI package. Complexity
210 of the networks, based on the average node degree, was different depending on the country
211 and on the diagnosis. For instance, Belgian networks and the Spanish PP network were simpler
212 than the rest, while Peruvian networks showed the higher level of complexity. Moreover, most
213 of the network connections were positive, suggesting a synergy between the bacterial genera
214 involved in the networks, with the exception of the genus *Streptococcus*, which suggested
215 antagonism, in the network of Peruvian PP, against genera related to periodontitis such as
216 *Porphyromonas, Treponema, Catonella, Campylobacter* and *Prevotella*, and against
217 *Fusobacterium* in the Peruvian network of HS. The average node degrees were 0.43 (SD ± 0.8),
218 1.14 (SD ± 1.06), 0.54 (SD ± 0.65), 0.95 (SD ± 1.04), 0.22 (SD ± 0.48), 1.55 (SD ± 1.61), 0.85 (SD ±
219 0.89) and 0.25 (SD ± 0.59) for Belgian, Peruvian, Chilean and Spanish HS, and Belgian, Peruvian,
220 Chilean and Spanish PP, respectively. Betweenness among nodes was similar, averaging 0.55 (SD
221 ± 0.64), with the exception of the Peruvian PP network, which showed a significantly ($p = 0.014$)
222 higher betweenness of 13.1 (SD ± 32.6). No major differences were observed regarding the
223 closeness of the nodes, averaging a value of 7.37×10^{-4} (SD ± 6.2×10^{-5}).

224

4. Discussion

In this study, we analysed the subgingival microbial profiles of 506 volunteers, 196 of whom were HS and 310 PP, recruited from 4 different countries in order to assess whether the geographic origin of the samples had an impact on the composition of the subgingival microbial profiles. Previous studies have attempted to clarify how the oral microbiome can shift depending on the geographical or ethnic origin of the subjects[9,15,29–35]. However, due to the variety of identification techniques and types of samples analysed, the low number of individuals enrolled in the studies and even the kind of analysis performed on the results, it is difficult to compare the outcomes of these studies and therefore to paint the big picture. To our knowledge, there is no previous study that has aimed to perform a metagenomic comparative analysis of the subgingival microbiota of both HS and PP from 4 different countries with such a large number of individuals using next generation sequencing techniques. This study showed that, despite there being a clear association between microbiota profile and subject diagnosis, the country of origin also plays a role in defining such profiles.

As previously described[36], genera that contain species associated with periodontitis, such as *Tannerella*, *Treponema*, *Filifactor* and *Porphyromonas*, were significantly more abundant in PP (Figure 4). Other genera whose association with periodontitis has been poorly studied were also significantly more abundant in PP, such as the genera *Desulfovibrio, Fretibacterium, Oceanivirga and Odoribacter,* among others. From these, it is worth noting *Fretibacterium*, which was first described in 2013[37]. Recent studies[38–40] are building evidence on the association between this genus and periodontal disease, which might be on a par with classic periodontopathogens such as *P. gingivalis* or *T. denticola*. However, more evidence on its role in periodontitis is still needed and might be difficult to obtain due to its strict *in vitro* growth requirements[41].

We were able to identify a microbial core associated with periodontitis, composed of the genera to which the bacteria of the red-complex[42] pertain, together with the previously mentioned genus *Fretibacterium* and the genus *Filifactor*, which has also seen growing evidence of its relationship with periodontitis and other oral infections[16,43]. Likewise, a healthy core microbiome was detected, including bacteria from the genera *Actinomyces, Haemophilus, Rothia, Cardiobacterium* and *Bergeyella,* among others, which have been previously associated with periodontal health[6,44] (Table 2). When looking at the core microbiomes of HS in each country, some differences were observed between the most significantly associated genera, while such differences were scarcer in the microbial cores of PP.

A plausible explanation for this observation might come from the hypothesis regarding pathogenic microbial communities[45], where individual microorganisms play a limited role in the development of the disease, and it is the pathogenic community of microorganisms that has a greater and more relevant impact. Assuming this, it is more plausible for a common pathogenic microbial community to be preserved, even among different geographical locations. On the other hand, polymicrobial synergy in health might allow for a higher variability in the microbial communities[9,10,46], which might be shaped according to the cultural and demographic differences that exist between countries and/or ethnicities[9,47,48].

Overall, microbial richness was significantly higher in PP of all countries with the exception of Spain, which did not show significant differences (Figure 1 and Table S1). On the other hand, although diversity values were constantly higher in PP, significant differences were only detected in Peruvian samples. The increase in richness and diversity in PP has been previously described[49,50], which might be due to the incorporation to the subgingival biofilm of the late colonisers, increasing the number of species and reducing the hegemony of certain commensal bacteria, thus increasing richness and diversity. Moreover, the levels of richness and diversity were significantly different between countries, particularly when comparing Peruvian PP with the PP of the other countries. Here, as has been previously observed in the gut and oral microbiome[51–53], cultural differences, such as dietary habits, might be of great importance to understand such differences, given that Chile is one of the most westernised countries of South America, and Spain and Belgium are fully westernised European countries[54].

From all the variables analysed in this study, country of origin and subject diagnosis constantly accounted for significant differences in the composition of the subgingival microbiota for all the comparisons studied. Furthermore, the country of origin of the subjects accounted for significant differences in the composition of the subgingival microbiota in the HS group but not in the PP group (Table S2). The amount of contribution of subject diagnosis to the variation of the microbial composition was much larger than country of origin. However, this contribution was unequal among the countries, with a more than fourfold larger impact on Belgian samples (42.13% of the variation explained) than on Peruvian samples (9.1%). The scarce variability in the microbiota explained by diagnosis in Peru, which defies the common consensus that diagnosis is able to define the microbiota to a high degree[6,55], highlights the bias that instead of taking into account underrepresented ethnicities or cultures, most studies base their results mainly on westernised subjects[36]. On the other hand, the demographic variables and the clinical outcomes of the subjects did not have a significant impact on the microbial composition of the samples on their own, regardless of their country of origin or their diagnosis, suggesting that

these variables should not be taken as predictors, on their own, of microbial profiles associated with either a healthy status or with periodontitis.

Network analysis, based on the co-occurrence of ASVs, allowed us to determine which taxa experienced a potential synergism or antagonism. From these networks we could observe that most of the significant relationships were positive, indicating that certain genera thrive in the same environmental conditions or thanks to other genera. Specifically, we could observe how most of the commensal bacteria developed a positive relationship with one another, both in HS and in PP. The same happened with genera associated with periodontitis, suggesting that even in HS, these microorganisms are clustered whether due to the same growth requirements, metabolic interactions or to some level of mutualism, as has been previously described[56,57]. Moreover, although scarce, some antagonist relationships were detected in the Peruvian samples, where the genus *Streptococcus* showed a negative co-occurrence with late colonisers such as *Fusobacterium, Campylobacter, Prevotella* or *Porphyromonas*. Given that some of these relationships have been considered as positive in the past[58,59], such as the relationship between *P. gingivalis* and *Streptococcus gordonii*[57], further studies should be conducted in order to better understand these links, taking into account that more profound knowledge of subgingival bacterial interactions could lead to the establishment of an eubiotic subgingival biofilm through the use of probiotics[60,61].

Our study is not exempt of limitations: HTS based on the V3-V4 regions of the 16S rRNA gene is not able to assign species level taxonomy to the ASVs with enough confidence[62], and therefore resolves at the genus level, at best. This lack of resolution might be solved using costlier techniques such as the whole genome sequencing or third-generation sequencing approaches, or the narrower but more specific approach of quantitative PCR with specific primers and probes. Moreover, despite the fact that all centres from the 4 countries were instructed to follow the same inclusion and exclusion criteria, clinicians were not calibrated, which might account for some of the differences observed in the clinical outcomes in the different countries. Furthermore, inclusion and exclusion criteria were based mainly on subject diagnosis, without taking into account other variables such as age, gender, sampling site, the hour in which the sample was collected, the time passed since the last food intake or the clinical outcomes, among others, adding to the heterogeneity of the subjects studied. However, the large number of samples were meant to mitigate such diversity, as was observed in the microbial profiles when clustering the samples by diagnosis (Figures 2 and S2).

In conclusion, our study shows that the subgingival microbiome can change significantly according to the geographical origin of the subjects, whether healthy or diseased. Nevertheless, from the variables analysed in this study, subject diagnosis has the highest weight in terms of defining the subgingival microbiota. Moreover, a highly conserved core of bacterial genera associated with periodontitis was detected in all the countries, while genera associated with a healthy status were more varied. Further studies using techniques with a higher resolution might be needed to confirm and expand the findings of this work. However, the differences observed in this study in richness, diversity, and composition of the subgingival microbiota of different geographical locations highlight the importance of analysing microbial profiles all over the globe in order to reduce biases in the field of oral metagenomics which, in the end, might improve future treatments and diagnostic strategies.

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Author's contribution

Conceptualisation: RL and VB; **Methodology**: GA, RL and VB; **Resources:** CM, EH, GM, IL, JN, LI, MJC, WT; **Investigation**: GA and SI; **Formal analysis**: AA; **Visualisation**: AA; **Writing – original draft preparation**: AA; **Writing – review and editing**: AA, CM, EH, GA, GM, IL, JN, LI, MJC, RL, SI, VB, WT.

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Table 1. Clinical and demographic characteristics of the subjects enrolled. Significant differences ($p < 0.05$) between groups of each variable are noted with the same super index letter. Comparisons were made between HS and PP, between countries without considering the diagnosis, and between all the HS and PP groups of each country. Super index letters are not related between different columns. HS: healthy subjects, PP: periodontitis patients, BOP: bleeding on probing, CAL: clinical attachment level, PPD: periodontal pocket depth.

Groups	N	Age (mean ± CI95)	Gender M (%)	BOP yes (%)	CAL (%)			PPD (%)		
					≤ 3 (mm)	3 - 5 (mm)	≥ 5 (mm)	≤ 3 (mm)	3 - 6 (mm)	≥ 6 (mm)
Total	506	45.2 ± 1.2	49.01	52.37	41.08	15.23	43.69	38.4	37.4	34.2
Healthy	196	37.3 ± 1.5^{l}	42.35	20.92^{f}	96.95^{f}	3.02^{a}	0^{a}	91.88^{a}	8.12^{a}	0^{a}
Periodontitis	310	50.3 ± 1.5^{l}	53.23	72.26^{f}	4.64^{f}	23.18^{a}	72.19^{a}	3.63^{a}	56.44^{a}	39.94^{a}
Belgium	76	36.3 ± 3.5$^{g, h}$	47.37	55.26	55.07	1.45$^{b, c, d}$	43.48	46.38	15.94$^{b, c, d}$	37.68^{b}
Belgium HS	38	24.5 ± 1.1$^{a, b, c, m}$	47.37	28.95	100^{a}	0	0^{e}	84.21$^{c, d}$	15.79$^{g, h}$	0^{j}
Belgium PP	38	50.8 ± 3.3^{m}	47.37	81.58	0^{a}	3.26$^{h, j}$	96.77$^{c, e}$	0	16.13$^{k, l, m}$	83.87$^{f, g, j}$
Chile	81	45.9 ± 2.5$^{g, i, k}$	37.04	69.14	48.15	11.11$^{d, f}$	40.74	47.56^{b}	30.49$^{d, f}$	21.95$^{d, e}$
Chile HS	38	39.9 ± 2.4$^{a, p}$	28.95	36.84^{d}	100^{d}	0^{m}	0^{h}	100$^{d, f, l}$	0$^{h, j, s}$	0^{l}
Chile PP	43	51.1 ± 3.5$^{f, p}$	44.19	97.67	0^{d}	21.43^{m}	78.57^{h}	0^{l}	58.14$^{m, o, p, s}$	41.86$^{g, i, l}$
Peru	172	41.6 ± 2.9$^{j, k}$	50.58	62.79^{a}	36.63	27.91$^{c, e, f}$	35.47^{b}	36.63	59.88$^{c, e, f}$	3.49$^{b, c, e}$
Peru HS	64	37.3 ± 2.9$^{c, d, o}$	54.69	15.62^{c}	90.62$^{c, e}$	9.38^{g}	0^{g}	98.44$^{c, e, k}$	1.56$^{g, i, r}$	0
Peru PP	108	44.2 ± 3.0$^{e, f, o}$	48.15	90.74$^{c, e}$	4.63^{c}	38.89$^{j, k}$	56.48$^{c, d, g}$	0$^{g, k}$	94.44$^{l, n, p, r}$	5.56$^{f, h, i}$
Spain	177	51.8 ± 1.7$^{h, i, j}$	53.67	33.33^{a}	36.72	10.17$^{b, e}$	53.11^{b}	32.77^{b}	27.12$^{b, e}$	40.11$^{c, d}$
Spain HS	56	44 ± 1.9$^{b, d, n}$	33.93^{a}	10.71$^{b, d}$	100$^{b, e}$	0$^{g, l}$	0^{f}	83.93$^{e, f}$	16.07$^{i, j, q}$	0^{k}
Spain PP	121	55.4 ± 1.9$^{e, n}$	62.81^{a}	43.8$^{b, e}$	7.44^{b}	14.88$^{h, k, l}$	77.69$^{d, f}$	9.09^{g}	32.23$^{k, n, o, q}$	58.68$^{h, k}$

Table 2. Top 5 bacterial genera linked to either subject diagnosis, country of origin or both, sorted by the statistical significance of their association and with the p value between parentheses.

By diagnosis	
Healthy subjects	**Periodontitis patients**
Actinomyces (0.0001)	*Tannerella* (0.0001)
Haemophilus (0.0001)	*Fretibacterium* (0.0001)
Rothia (0.0001)	*Filifactor* (0.0001)
Cardiobacterium (0.0001)	*Treponema* (0.0001)
Bergeyella (0.0001)	*Porphyromonas* (0.0001)

By country – Healthy subjects			
Belgium	**Chile**	**Peru**	**Spain**
Streptococcus (0.0001)	*Actinomyces* (0.0001)	*Alkalibacterium* (0.0001)	*Streptococcus* (0.0001)
Actinomyces (0.0001)	*Pseudomonas* (0.0001)	*Erysipelothrix* (0.0001)	*Haemophilus* (0.0001)
Corynebacterium (0.0001)	*Rothia* (0.0001)	*Rothia* (0.0001)	*Bergeyella* (0.0001)
Granulicatella (0.0001)	*Cutibacterium* (0.0001)	*Anaerobacillus* (0.0001)	*Gemella* (0.0001)
Rothia (0.0001)	*Pseudopropionibacterium* (0.0001)	*Tessaracoccus* (0.0001)	*Rothia* (0.0001)

By country – Periodontitis patients			
Belgium	**Chile**	**Peru**	**Spain**
Fretibacterium (0.0001)	*Fretibacterium* (0.0001)	*Fretibacterium* (0.0001)	*Fretibacterium* (0.0001)
Tannerella (0.0001)	*Porphyromonas* (0.0001)	*Peptostreptococcus* (0.0001)	*Filifactor* (0.0001)
Treponema (0.0001)	*Tannerella* (0.0001)	*Tannerella* (0.0001)	*Tannerella* (0.0001)
Filifactor (0.0001)	*Treponema* (0.0001)	*Treponema* (0.0001)	*Rikenellaceae RC9* gut group (0.0001)
Family XIII UCG-001 (0.0001)	*Filifactor* (0.0001)	*Filifactor* (0.0001)	*Porphyromonas* (0.0001)

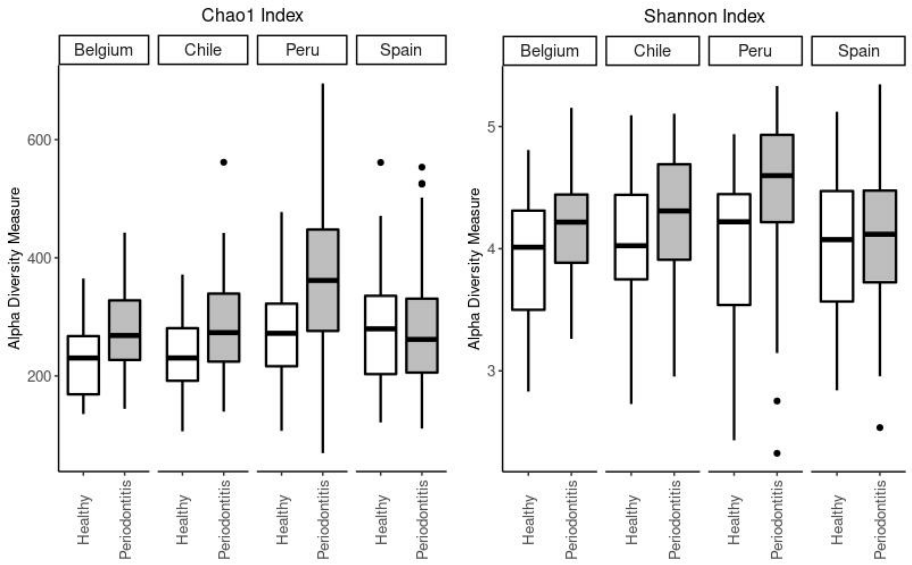

Figure 1. Richness (Chao1 index) and diversity (Shannon index) of the microbiota obtained from healthy subjects and periodontitis patients, grouped by their country of origin.

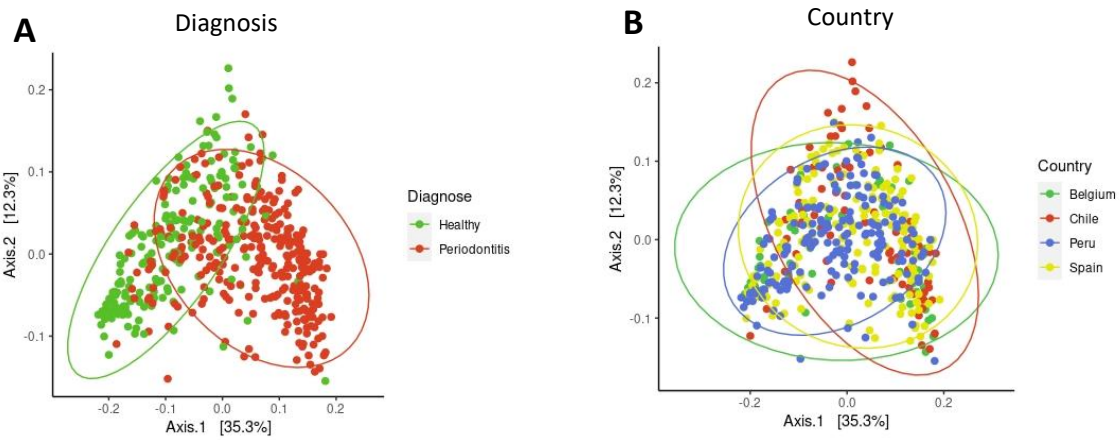

Figure 2. Principal Coordinates Analysis (PCoA) of weighted UniFrac plots of the microbiome structure of the subgingival samples, grouped by their periodontal health status (A) or their country of origin (B).

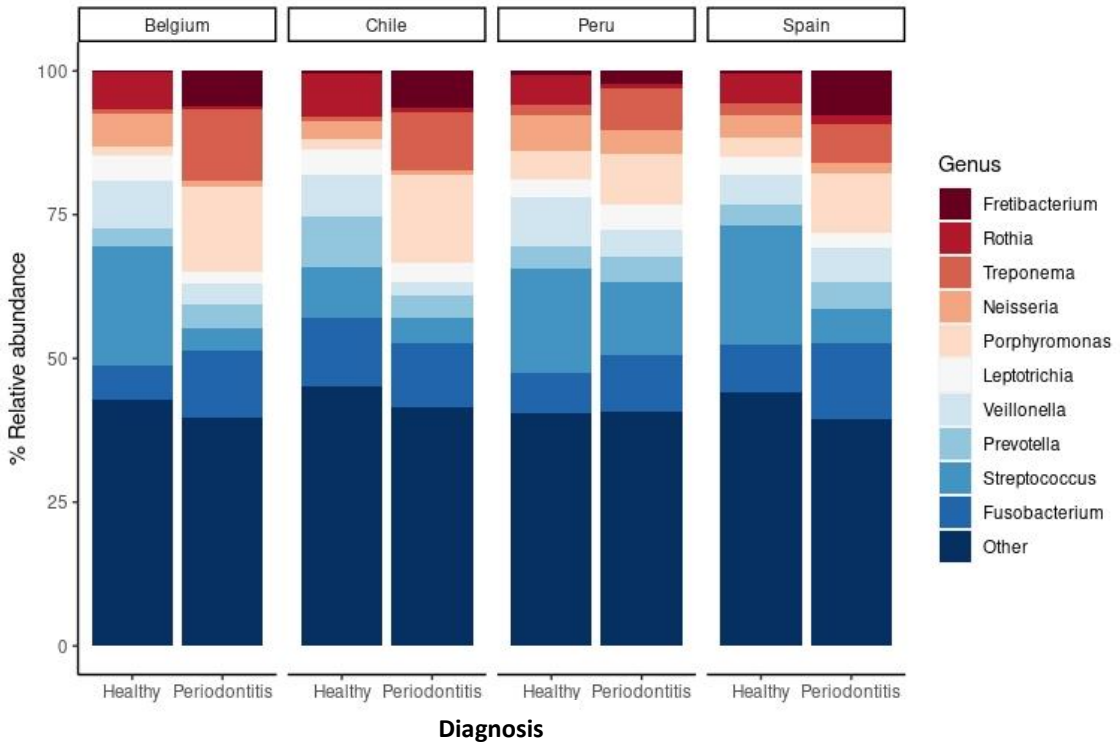

Figure 3. Relative abundance of the top 10 bacterial genera identified in the study subjects, separated by the subjects' country of origin and diagnosis. The label Other includes the taxa not included in the 10 most abundant genera.

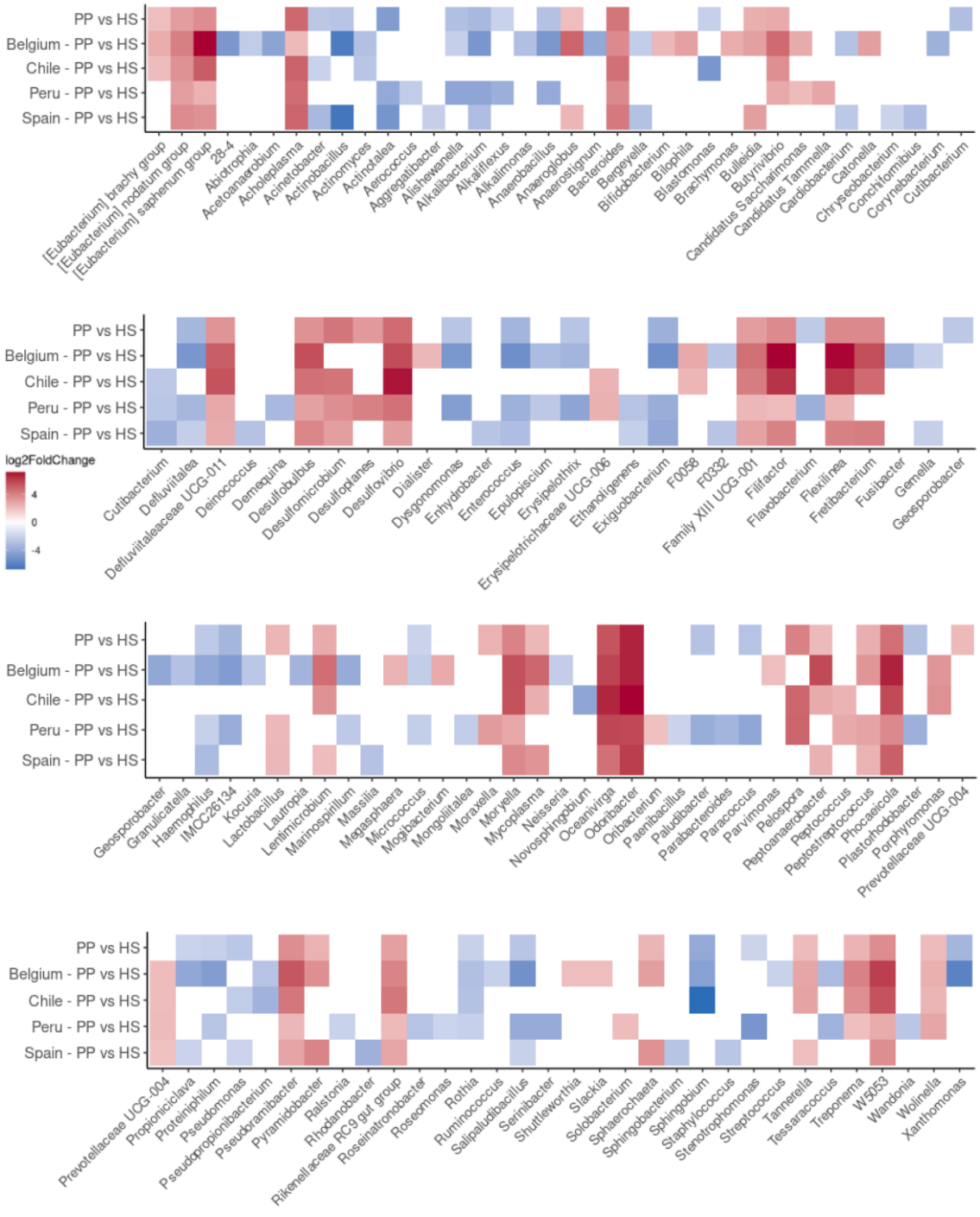

Figure 4. Heatmap of the differential abundance of bacterial genera according to subject diagnosis and country of origin

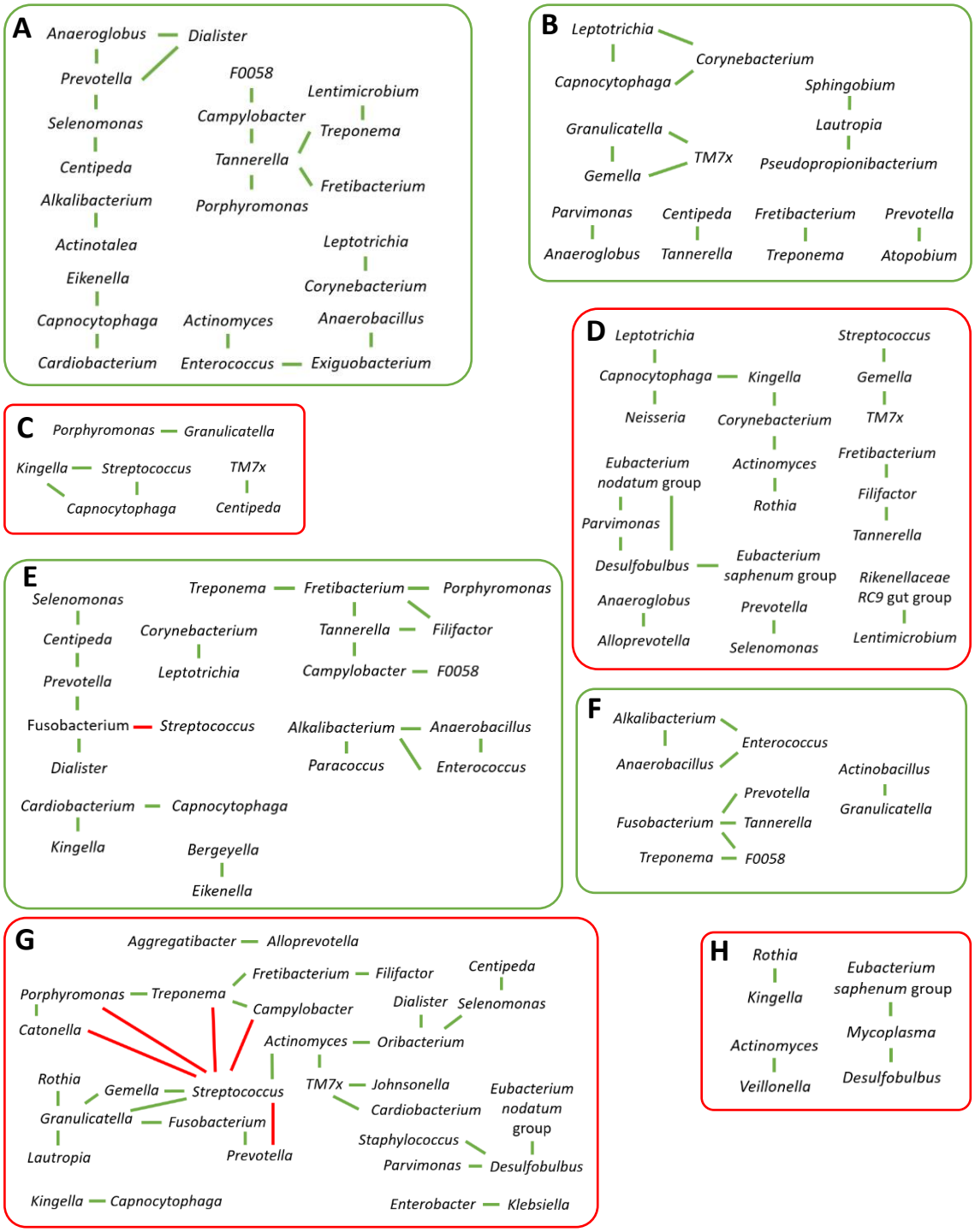

Figure 5. Microbiome networks, showing how the relative abundance of certain bacterial genera were influenced, positively or negatively, in the presence of other genera. A green line indicates a positive relationship, while a red line indicates a negative relationship. In order to make the plot readable, genera were filtered by representing at least a 3% of relative abundance, and only those that showed interactions are displayed. Populations surrounded by a green rectangle are healthy subjects, while those surrounded by a red rectangle are periodontitis patients. A) Spain healthy, B) Chile healthy, C) Spain periodontitis, D) Chile periodontitis, E) Peru healthy, F) Belgium healthy, G) Peru periodontitis and H) Belgium periodontitis.

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