

RESEARCH ARTICLE

Gene expression of *Porphyromonas gingivalis* ATCC 33277 when growing in an *in vitro* multispecies biofilm

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Abstract

Background and objective

Porphyromonas gingivalis, an oral microorganism residing in the subgingival biofilm, may exert diverse pathogenicity depending on the presence of specific virulence factors, but its gene expression has not been completely established. This investigation aims to compare the transcriptomic profile of this pathogen when growing within an *in vitro* multispecies biofilm or in a planktonic state.

Materials and methods

P. gingivalis ATCC 33277 was grown in anaerobiosis within multi-well culture plates at 37°C under two conditions: (a) planktonic samples (no hydroxyapatite discs) or (b) within a multi-species-biofilm containing *Streptococcus oralis*, *Actinomyces naeslundii*, *Veillonella parvula*, *Fusobacterium nucleatum* and *Aggregatibacter actinomycetemcomitans* deposited on hydroxyapatite discs. Scanning Electron Microscopy (SEM) and Confocal Laser Scanning Microscopy (CLSM) combined with Fluorescence *In Situ* Hybridization (FISH) were used to verify the formation of the biofilm and the presence of *P. gingivalis*. Total RNA was extracted from both the multispecies biofilm and planktonic samples, then purified and, with the use of a microarray, its differential gene expression was analyzed. A linear model was used for determining the differentially expressed genes using a filtering criterion of two-fold change (up or down) and a significance p-value of <0.05. Differential expression was confirmed by Reverse Transcription-quantitative Polymerase Chain Reaction (RT-qPCR).

Results

SEM verified the development of the multispecies biofilm and FISH confirmed the incorporation of *P. gingivalis*. The microarray demonstrated that, when growing within the multispecies biofilm, 19.1% of *P. gingivalis* genes were significantly and differentially expressed (165 genes were up-regulated and 200 down-regulated), compared with planktonic growth. These genes were mainly involved in functions related to the oxidative stress, cell envelope, transposons and metabolism. The results of the microarray were confirmed by RT-qPCR.

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Data Availability Statement: Gene expression data can be found at the Gene Expression Omnibus (GEO) repository using the accession GSE132157. Please see GEO data at the following URL: <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE132157>.

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Conclusion

Significant transcriptional changes occurred in *P. gingivalis* when growing in a multispecies biofilm compared to planktonic state.

Introduction

The oral cavity is a unique ecological environment colonized by more than 500 bacterial species [1–3]. These bacteria are part of the oral microbiome and may be floating freely or within structured bacterial communities being part of complex biofilms, which provide bacteria protection against shear forces and host immune responses [4–6]. If these biofilms are not allowed to grow and mature, mainly through effective oral hygiene practices, these stable communities develop immune tolerance and may remain in symbiosis with the oral tissues. However, if they increase in mass or there are relevant changes in the local environment that favors the growth of pathobionts (dysbiosis), the immunological tolerance will be surpassed leading to inflammation [7, 8]. Among these pathobionts, *Porphyromonas gingivalis* has shown the expression of virulence factors to evade the host responses and to favor its colonization and spread within the tissues [9, 10].

Several studies have shown that when *P. gingivalis* grows within a biofilm, specific genes will become differentially regulated [11–14]. These genes may be relevant in promoting phenotypic adaptations of this pathogen, what may facilitate its infective potential [15–17], mostly by evading the immune response and promoting non-resolving chronic inflammation resulting in soft and hard tissue destruction, which are the key pathological features of periodontitis [5, 9, 18–20]. There is, however, scarce transcriptomic information of *P. gingivalis* and most available knowledge on the gene expression comes from *in vitro* monospecies biofilm models [21, 22]. Our research group has recently reported significant differences of gene expression when *P. gingivalis* was growing within a monospecies biofilm, mainly in those genes related to cell envelope, transport, outer membrane proteins, transposases and oxidative stress genes [14]. Furthermore, significant differences were encountered in those genes related to metabolism, adhesion, invasion, virulence and quorum sensing, when a growing monospecies biofilm was in the presence of planktonic *P. gingivalis* [23]. However, within the oral cavity, symbionts and pathobionts colonize as multispecies biofilms [7, 19, 24–26], and these bacteria will be faced with diverse DNA exchanges (horizontal gene transfer) and to multiple stressors, but only those bacteria with expressed genes that will enable them to colonize or resist host defenses, will be able to survive and predominate [2, 3, 27–30]. It was, therefore, the purpose of this *in vitro* study to compare the gene expression of *P. gingivalis* when growing within a multispecies oral biofilm with its growth in planktonic conditions.

Material and methods

Bacterial strains and culture conditions

Methodology for developing the multispecies biofilm was similar to that previously reported from our research group [14, 23]. Briefly, reference strains of *P. gingivalis* ATCC 33277, *Streptococcus oralis* CECT 907T, *Actinomyces naeslundii* ATCC 19039, *Veillonella parvula* NCTC 11810, *Fusobacterium nucleatum* DMSZ 20482 and *Aggregatibacter actinomycetemcomitans* DSMZ 8324 were used. Each bacterial strain was grown on blood agar plates (blood agar Oxoid no. 2; Oxoid, Basingstoke, UK), supplemented with 5% (v/v) sterile horse blood

EXPERIMENTAL DESIGN

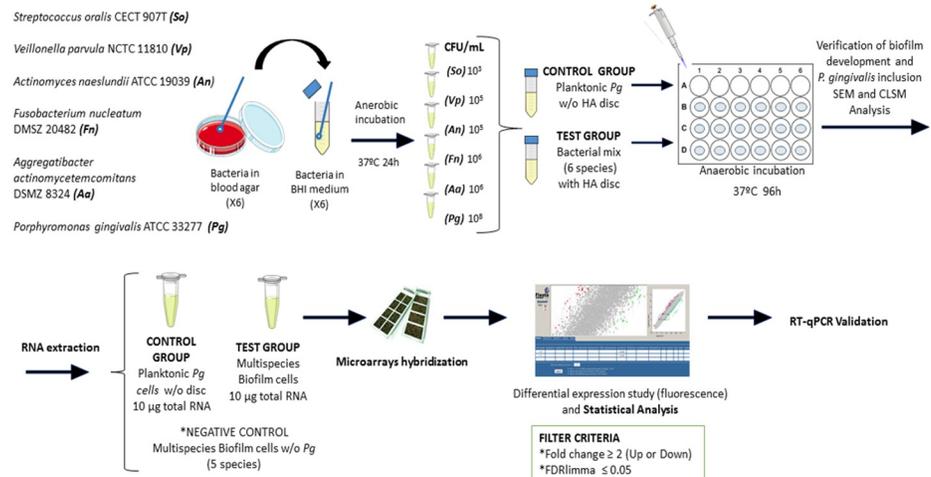


Fig 1. Overview of experimental design. *P. gingivalis* ATCC 33277 was incubated in anaerobiosis at 37°C for 96 h and grown under planktonic conditions (control group) and within a multispecies-biofilm on hydroxyapatite (HA) discs, also containing the other described five bacterial species (test group). Scanning Electron Microscopy (SEM) and Confocal Laser Scanning Microscopy (CLSM) were used to verify the multispecies-biofilm development and the presence of *P. gingivalis* in it. Total RNA was extracted, purified and the differential gene expression was analyzed by microarray [Agilent *P. gingivalis* Oligo Microarrays 8x15K (074976)] with a filtering criterion of two-fold change (up or down) and significance p-value < 0.05. Differential expression was confirmed by Reverse Transcription-quantitative Polymerase Chain Reaction (RT-qPCR). (Images for Fig 1 were taken from <https://smart.servier.com/> under a creative commons license).

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(Oxoid), 5.0 mg/L hemin (Sigma, St Louis, MO, USA) and 1.0 mg/L menadione (Merck, Darmstadt, Germany) under anaerobic conditions (10% H₂, 10% CO₂ and 80% N₂) at 37°C for 24–72 h.

Experimental assays

Fig 1 depicts the experimental design of this investigation. Planktonic cultures of each reference strain were grown anaerobically at 37°C for 24 h in a protein-rich medium containing Brain-Heart Infusion (BHI) (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) supplemented with 2.5 g/L mucin (Oxoid), 1.0 g/L yeast extract (Oxoid), 0.1 g/L cysteine (Sigma), 2.0 g/L sodium bicarbonate (Merck), 5.0 mg/L hemin (Sigma), 1.0 mg/L menadione (Merck) and 0.25% (v/v) glutamic acid (Sigma). By means of spectrophotometry, the late exponential phase growth was verified (optical density at 550 nm). Due to the fact that the bacteria used have different growth rates, and that this biofilm model is static we have adjusted the inocula of the different bacteria as was previously developed and validated by Sánchez *et al.*, [31], in order to avoid the overgrowth of certain species and an excessive accumulation of waste products, obtaining by dilution in fresh modified BHI medium the following final concentrations:

- 10³ colony forming units (CFUs)/mL for *S. oralis*,
- 10⁵ CFUs/mL for *V. parvula* and *A. naeslundii*,
- 10⁶ CFUs/mL for *F. nucleatum* and *A. actinomycetemcomitans*,
- 10⁸ CFUs/mL for *P. gingivalis*.

Using pre-sterilized polystyrene 24-well tissue culture plates (Greiner Bio-one, Frickenhausen, Germany), two types of growing conditions were developed:

1. The test group (*P. gingivalis* growing within a multispecies biofilm). In each well of the plate, 1.5 mL of the mix solution containing the six reference strains, at the above referred concentrations, were deposited together with a sterile ceramic calcium hydroxyapatite discs (HA) [7-mm diameter (standard deviation, SD = 0.2 mm) and 1.8 mm thickness (Clarkson Chromatography Products, Williamsport, PA, USA)];
2. The control group (*P. gingivalis* growing planktonically). In each well, a volume of 1.5 mL of pure culture of *P. gingivalis* (10^8 CFUs/mL) was deposited in the absence of discs.

The plates were then incubated in anaerobiosis at 37°C for 96 h. To rule out any possible contamination, a set of wells within the same plate were incubated with only culture medium.

Monitoring of biofilm development: Scanning electron microscope and confocal laser scanning microscopy

In order to verify the multispecies-biofilm development on discs, Scanning Electron Microscope (SEM) was used. Three HA discs covered with biofilms grown for 96 h were fixed in 4% paraformaldehyde and 2.5% glutaraldehyde for 4h at 4°C, then washed twice in phosphate-buffered saline (PBS) and sterile water (immersion time 10 min) and dehydrated through a series of graded ethanol solutions (50, 60, 70, 80, 90 and 100%; immersion time per series, 10 min). Then, critical point drying and sputter-coating with gold was carried out before analyzing the samples with a scanning electron microscope JSM 6400 (JSM6400; JEOL, Tokyo, Japan) equipped with back-scattered electron detector and with an image resolution of 25 KV.

In addition, in order to ensure the incorporation of *P. gingivalis* from planktonic to biofilm, Confocal Laser Scanning Microscopy (CLSM) combined with Fluorescence *In Situ* Hybridization (FISH) was used. Three HA discs covered with multispecies biofilms, grown *in vitro* for 96 h, were incubated for 18 h with 40 µg/ml in hybridization buffer of the 16S rRNA *P. gingivalis* ALEXA Fluor 488 probe [5′-3′: CACTGAACTCAAGCCCGGCAGTTTCAA; Life Technologies Invitrogen (Carlsbad, CA, USA)]. Stained biofilms were washed for 15 min in a wash buffer [0.1 M Tris-HCl [pH 7.2], 0.18 M NaCl, 0.05 M EDTA and 0.005% sodium dodecyl sulfate [wt/vol]], and then exposed to 1 µg/mL of DAPI (4′,6-Diamidino-2-Phenylindole, Dihydrochloride; Thermo Fisher Technologies, Life Technologies Corporation, Carlsbad, CA, USA) for 5 min. Specimens were then washed 10 min with wash buffer and examined with a fixed-stage Ix83 Olympus inverted microscope coupled to an Olympus FV1200 confocal system (Olympus; Shinjuku, Tokyo, Japan). The objective lens was a ×63 water-immersion lens (Olympus) and image stacks were acquired with a z-step size of 0.5 µm thickness (8 bits, 1024x1024 pixels). ALEXA Fluor 488 signals were detected with a PMT detector using a 405 nm laser and an emission range of 647–665 nm, together with DAPI (PMT detector / 552 nm laser / 350–470 nm emission range). Image analysis was performed with Imaris Biteplane software (Belfast, UK).

Harvesting of planktonic and biofilm cells for gene expression analysis

After 96 h incubation in anaerobiosis at 37°C, samples from both groups, test and control, were harvested and pooled into three biological replicates of each condition for independent hybridization. In the control group, a total of 5 mL were recovered from the wells of the culture plates and sequentially centrifuged to pool them into a single pellet in order to obtain 10 µg of RNA. In the test group, a set of 100 discs per biological replicate were harvested independently in 1 mL of sterile PBS and disaggregated by vortex during 3 min. The disaggregated multispecies biofilms were then pooled in a single sample, to obtain at least 10 µg of total RNA per biological replicate.

Both samples groups (planktonic and multispecies biofilms) were processed in the same manner, centrifuged at 9,000 rpm at 4°C during 5 min and pooled as a single pellet for each biological replicate.

In all cases, an aliquot of each sample was used as quality control. For that, these aliquots were cultured on supplemented blood agar plates under anaerobic conditions at 37°C for two weeks to control for the presence of each intended bacteria and the absence of contamination.

Total RNA extraction

The three biological replicates of each condition, test and control, were suspended in 1 mL of TRIzol reagent (Ambion, NY, USA) to lysate the cells and to extract the total RNA [TRIzol Plus RNA Purification Kit (Invitrogen)]. Then, 200 µL of cold chloroform was added to separate its hydrophobic and hydrophilic content. The mixtures were then centrifuged at 13,000 rpm for 15 min at 4°C, and the RNA phase collected. Nucleic acids were then precipitated with ~ 500 µL of cold 70% ethanol and centrifuged at 11,300 rpm for 15 seconds at room temperature. Pellets were then suspended in 50 µL of RNase-free water (Roche Diagnostics, Mannheim, Germany). To ensure the absence of any contaminating DNA, DNase I (Ambion, NY, USA) was added to the samples (set of RNase-free DNase; Qiagen, CA, USA) and purified using columns of RNeasy Mini kit (Qiagen) following the manufacturer's protocol.

RNA quantity and quality were measured with Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). An A260/A280 ratio of at least 2.0 was reached for all the samples used in this study.

cDNA synthesis, labeling and hybridization

The three biological replicates were independently hybridized for transcriptomic comparison of the test and control groups. The fluorescent labeling was performed using SuperScript Indirect cDNA Labelling System (Invitrogen; Carlsbad, CA, USA). Preparation of probes and hybridization were performed following the manufacturer's instructions [One-Color Microarray Based Gene Expression Analysis Manual Ver. 6.5 (Agilent Technologies)]. A slide specific for the strains of *P. gingivalis* ATCC 33277 and W83 was used [Agilent *P. gingivalis* Oligo Microarrays 8x15K (074976)].

As negative control, two replicates of multispecies biofilm without *P. gingivalis* (5 species-biofilm) were prepared and loaded onto the same microarray to rule out any possible gene cross-hybridization from the other bacteria.

Microarray and data analysis

Images from Cy3 channel were equilibrated and captured with a high-resolution scanner (Agilent) and spots quantified using Feature Extraction software (Agilent) following a similar protocol as in the previous published investigations from our research group [14, 23]. Background correction and normalization of data expression were performed using LIMMA [32–34]. For local background correction and normalization, the methods "normexp" and loess in LIMMA were used, respectively [32]. To ensure consistency and similar distribution across arrays, log-ratio values were scaled using the median-absolute-value as scale estimator [33]. Linear model methods were used for determining differentially expressed genes. Each probe was tested for changes in expression over replicates by using an empirical Bayes moderated t-statistic [33]. To control for false discovery rates, p-values were corrected using the Benjamini and Hochberg method [32, 33]. We selected an expected false discovery rate of less than 5% and a filtering criterium of increase/decrease up to 2-fold differential expression between the two conditions, as used in other similar studies [14, 17]. Expression ratios were expressed as means of the fold changes of the three biological replicates and SD.

Table 1. Primers used for Reverse Transcription-quantitative Polymerase Chain Reaction (RT-qPCR).

LOCUS NAME	PUTATIVE IDENTIFICATION	PRIMERS FOR RT-qPCR	
PG_2131	OmpA_c-like	Forward Backward	5'–3': ACACACCCCTCTCGTCTGAG 5'–3': TCCCTTCCGGATAGCTCTG
PGN_0183 (<i>FimC</i>)	Minor component FimC	Forward Backward	5'–3': CCTTTTCAAGAAAGAAGCTTGAGGA 5'–3': GTCGGACTATCGGCTCGTT
PG_1712	Alpha-1,2-mannosidase family protein	Forward Backward	5'–3': GCTACGAAAGCCGTCATC 5'–3': GTACCACTCCCAACCTTTGC
PGN_1058 (<i>Ftn</i>)	Ferritin	Forward Backward	5'–3': GAAATGATCGAGGCTGTCGT 5'–3': GTCCTGTGATGCCATATCTCC
PGN_0033	Thioredoxin	Forward Backward	5'–3': CAACATTTGACGGCTTGGTA 5'–3': CCGATGTAGCCAGAAATCCA
PGN_1208 (<i>ClpB</i>)	ClpB protein	Forward Backward	5'–3': ACAAGGGGCATGTGGTAAAC 5'–3': AACCGAGGTTGACGTCAT

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The National Center for Biotechnology (Genomics Unit) at Autónoma University of Madrid (Spain) performed the hybridizations and statistical analysis.

The transcriptomic results were inspected manually using different Internet platforms KEGG (Kyoto Encyclopedia of Genes and Genomes) and UniProt.

Reverse Transcription-quantitative Polymerase Chain Reaction (RT-qPCR)

To validate microarray results, RT-qPCR of selected genes was performed, as described in our previous investigations [14, 23]. Three genes were selected from the up-regulated group and other three from the down-regulated group. Specific primers were designed using the Universal Probe Library Roche software tool, Roche Diagnostics (Table 1). *P. gingivalis* 16S rRNA gene was used as a reference gene for normalization of the RT-qPCR.

The cDNA was generated from 1 µg of total RNA using the High Capacity cDNA Archive Kit (Applied Biosystems, ThermoFisher Scientific) in a 10 µL of final reaction volume. Then qPCR reactions were performed in triplicate by using 5 µL per well of each cDNA, and 3 µL of a mix composed by 0.4 µM of each primer, 5x HOT FIREPol EvaGreen qPCR Mix Plus (ROX), and nuclease-free water, to reach a final volume of 8 µL in 384-well optical plates. PCR reactions were run in an Applied Biosystems ABI PRISM 7900HT machine with Software Defined Storage (SDS) v2.4 and standard protocol from Applied Biosystems (95°C 10 min, 40 cycles of 95°C 15 sec and 60°C 60 sec, and a final standard melting curve dissociation protocol).

All the RT-qPCR measurements were performed in triplicate and the results were analysed with the comparative cycle threshold method ($\Delta\Delta C_t$) [35]. The transcriptional \log_2 ratio from RT-qPCR analysis was plotted against the average \log_2 ratio values obtained by microarray analysis.

Results

A mature multispecies-biofilm was confirmed by SEM. Fig 2A depicts this multispecies-biofilm, where most of the bacteria were organized in clusters with *F. nucleatum* acting as the backbone inter-connecting among the other bacterial morphotype. The CLSM combined with FISH was applied to detect the presence of *P. gingivalis* within the mature multispecies-biofilm. Fig 2B depicts the presence of *P. gingivalis* highlighted with a fluorescent stain in purple among the rest of bacterial species stained nonspecifically with DAPI in blue.

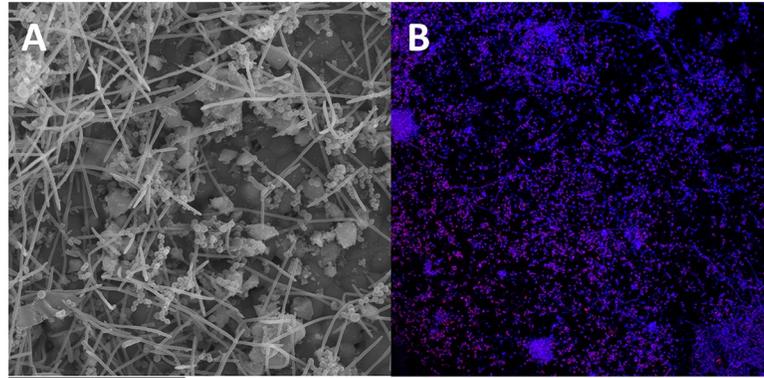


Fig 2. Micrographs representing the multispecies-biofilms after 96 hours of growth. (A) Scanning Electron Microscopy (SEM) depicting the structure of the biofilm. Note the microcolonies organized in clusters with *F. nucleatum* connecting them; (B) Confocal Laser Scanning Microscopy combined with Fluorescence *In Situ* Hybridization (CLSM-FISH). The ALEXA Fluor 488 probe detected the 16S rRNA *P. gingivalis* (cells in purple) within the multispecies-biofilm (other bacterial species in blue stained with DAPI).

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Fig 3 depicts the scanning glass slide of the microarray [Agilent *P. gingivalis* Oligo Microarrays 8x15K (074976)] and the fluorescence intensity measured after the hybridization of the three experimental replicates per group. Positive fluorescence was observed for *P. gingivalis* in planktonic growth (control group) (Fig 3 A1-A3) as well as within the multispecies biofilm (test group) (Fig 3 B1-B3). In contrast, there was no fluorescence in the negative control group, when *P. gingivalis* was not part of the multispecies biofilm (Fig 3 C1-C2), which confirmed the high specificity of the microarray without any sign of cross-hybridization.

Fig 4 depicts the *P. gingivalis* gene differential expression generated by the microarray (expressed as \log_{10} of fluorescence), when growing in planktonic (X-axis, control) or within a multispecies biofilm (Y-axis, test). Using a linear model (LIMMA) with a filtering criterion of two-fold change (up or down) and significance p-value <0.05 , a total of 365 out of 1,909 genes (19.1%) were found to have a differential expression when the two growing conditions were compared (S1 Table).

The *P. gingivalis* differentially expressed genes could be categorized in functional groups as depicted in Fig 5.

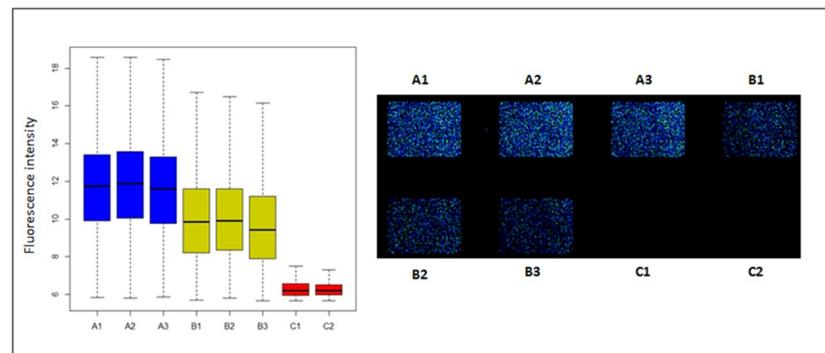


Fig 3. Fluorescence signal of hybridization conditions on the microarray represented in boxplot (left) and the microarray slide (right). Control group (A1, A2 and A3 in both cases) corresponds to the three experimental replicates of planktonic *Porphyromonas gingivalis* ATCC 33277. Test group (B1, B2 and B3 in both cases) represents the multispecies biofilm including *P. gingivalis*. And the negative control group (C1 and C2 in both cases) shows the multispecies-biofilm without *P. gingivalis*.

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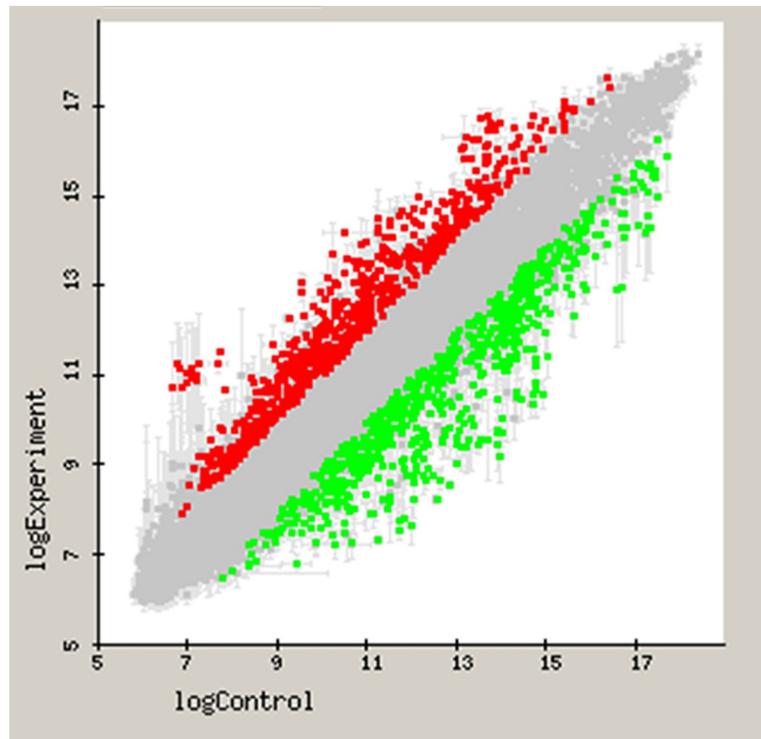


Fig 4. Microarray-based comparative transcriptome demonstrating the gene expression (represented in log₁₀) for *Porphyromonas gingivalis* ATCC 33277 when growing in a multispecies biofilm compared to planktonic growth. X-axis depicts the fold difference in gene expression of *P. gingivalis* in planktonic growth, and the Y-axis the gene expression of *P. gingivalis* inside a multispecies-biofilm. Up-regulated genes (over-expressed in multispecies-biofilm) were represented as red color and down-regulated genes were colored in green.

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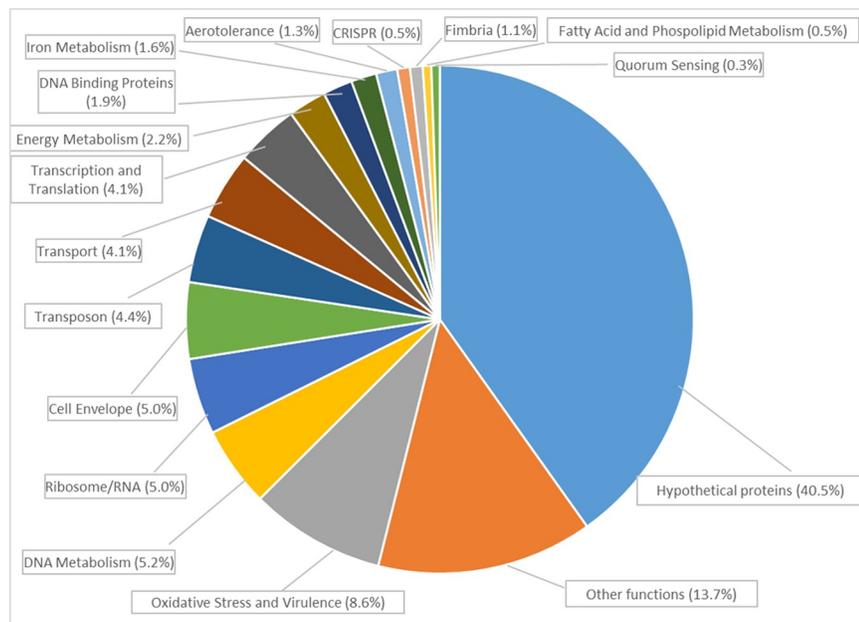


Fig 5. Distribution in functional categories of the differentially regulated genes of *Porphyromonas gingivalis* ATCC 33277 in planktonic cells compared to *P. gingivalis* within a multispecies-biofilm.

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The complete list of 365 genes can be found in [S1 Table](#). Of them, when *P. gingivalis* grew within a multispecies biofilm (test group), 165 genes were identified as up-regulated. These genes were mainly related to:

- **oxidative stress protection and secretion of virulence factors**, such as *SodB* superoxide dismutase [+5.71 (SD = 2.33)], thioredoxin system PGN_0033 [+7.33 (SD = 1.03)] and PG_0275 [+3.54 (SD = 1.11)], thiol peroxidases PG_1729 [+5.04 (SD = 0.74)] and PGN_0388 [+4.75 (SD = 1.01)], heat shock proteins as PGN_0041 [+2.58 (SD = 0.25)] and *Clp* system PGN_1208 [+8.31 (SD = 1.75)] and PGN_0008 [+3.01 (SD = 0.24)], chaperones *GroES* PGN_1451 [+7.93 (SD = 0.94)] and *GrpE* PGN_1715 [+2.95 (SD = 0.50)];
- **cell-cell communication**, such as the gene encoding for quorum sensing S-adenosylmethionine synthase (PGN_1827) [+2.55 (SD = 0.26)];
- **iron metabolism**, as ferritin PGN_1058 [+6.11 (SD = 1.48)] and PGN_0604 [+7.28 (SD = 1.54)] and ferredoxin PG_1813 [+2.25 (SD = 0.24)], *HmuY* [+3.29 (SD = 0.49)], PGN_0741 [+2.41 (SD = 0.38)];
- **ribosomes**, as *RpsA*, *RpsP*, *RpsT*, *RpmF*, *RpmH*, *RpmL*, *RplQ*, *RplT*, PG_0627 [+2.47 (SD = 0.05)] and PGN_0668 [+2.42 (SD = 0.17)];
- Other important functional genes, like the **transposon genes** that allow for adaptation to life in communities (*TraA-Q*) and CRISPR (*Cas2-2* PGN_1959 [+4.80 (SD = 0.50)]).

When *P. gingivalis* grew within a multispecies biofilm (test group), 200 genes were down-regulated ([S1 Table](#)). These genes were mainly related to:

- **cell envelope**, as membrane proteins PG_0922 [-7.29 (SD = 2.61)], PG_1180 [-2.48 (SD = 0.59)], PG_2224 [-5.33 (SD = 0.13)], PGN_1020 [-4.36 (SD = 0.83)];
- **lipoproteins**, as PG_0180 [-3.44 (SD = 0.56)], PG_0399 [-2.34 (SD = 0.29)], PG_2133 [-14.42 (SD = 3.64)], PG_0924 [-5.03 (SD = 0.80)];
- **transport**, as ABC transporters PG_0912 [-2.36 (SD = 0.05)], PGN_1898 *MgtE* [-2.24 (SD = 0.27)], PGN_1876 [-2.41 (SD = 0.18)], PG_1010 [-2.57 (SD = 0.23)], PGN_1343 [-2.81 (SD = 0.71)], PGN_1734 [-3.09 (SD = 0.43)];
- **aerotolerance**, as (*BatA-E*), PGN_0529 [-5.86 (SD = 1.09)], PGN_0528 [-5.15 (SD = 1.70)], PGN_0527 [-5.25 (SD = 0.65)], PGN_0526 [-4.74 (SD = 1.14)], PGN_0525 [-8.28 (SD = 0.63)];
- **fimbria**, as *FimA* [-11.03 (SD = 1.71)], *FimC* [-17.73 (SD = 2.28)], *FimD* [-9.65 (SD = 1.85)].

Of the 365 differentially regulated *P. gingivalis* genes, 40.5% encoded for unknown function or hypothetical proteins ([S1 Table](#)).

RT-qPCR confirmed the microarray results in three of the highly up-regulated genes and three of the highly down-regulated. [Fig 6](#) depicts the log₂ expression ratios for each technique demonstrating a high correlation between both ($R^2 = 0.9785$).

Discussion

The results from this *in vitro* investigation have revealed significant transcriptional changes when *P. gingivalis* grew within a multispecies biofilm, compared to planktonic growth, with 19.1% of *P. gingivalis* genes differentially expressed (165 genes were up-regulated and 200 down-regulated). The complete list of 365 genes can be found as Supporting Information file ([S1 Table](#)). In a previous report [14], we showed that gene expression of *P. gingivalis* changed

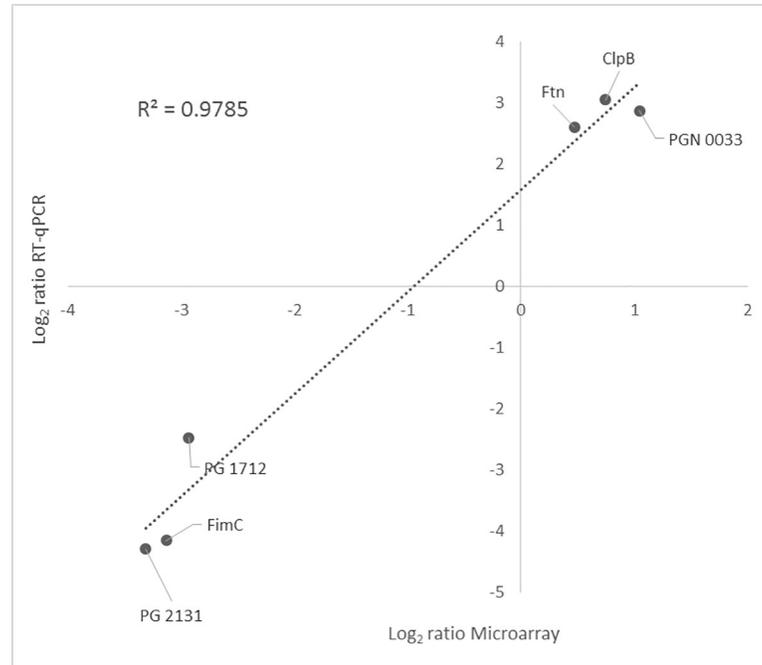


Fig 6. Correlation between Microarray and Reverse Transcription-quantitative Polymerase Chain Reaction (RT-qPCR) gene expression ratios when comparing *Porphyromonas gingivalis* ATCC 33277 growing within a multispecies-biofilm versus planktonic growth. RT-qPCR log₂ values were plotted against the microarray data log₂ values ($R^2 = 0.9785$).

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from free floating to sessile, but the present study demonstrates that when biofilm conditions become more complex, as it is a multispecies microbial community, gene expression changes significantly enhanced inside the functional categories. In agreement with previous reports [9, 36], this polymicrobial synergy may increase the ability of *P. gingivalis* to colonize/predominate in the presence of other bacteria and thus increase its pathogenicity [22].

When compared to planktonic growth, *P. gingivalis* within a multispecies biofilm had significant differential expression in relevant groups of genes related to different functions and pathogenic pathways:

Oxidative stress and virulence

The gene encoding the enzyme superoxide dismutase (*SodB*) was significantly up-regulated. This enzyme is responsible for transforming Reactive Oxygen Species (ROS) into peroxides, produced even in anaerobic conditions as a result of its own metabolism [37–39]. After that, peroxides are then generally disposed of by the enzyme catalase [40]. However, *P. gingivalis* cannot synthesize catalase and usually eliminates peroxides through the enzyme alkyl hydroperoxide reductase (*Ahp C–F*) [41]. Even though *AhpF* (PGN_0661) was down-regulated in the present study, the thioredoxin system (PGN_0033, PG_0275) and thiol peroxidase-encoding genes (PG_1729, PGN_0388), which are other pathways of peroxide removal [42, 43], were up-regulated. This finding has also been previously reported [44].

It was also significant with the up-regulation of genes involved in protein regulatory systems, such as heat shock proteins (Hsp), Clp proteolytic system (*ClpB*, *ClpC*) and the chaperones (*GroES*, *GrpE*), which are usually expressed during stresses situations [45]. Clp proteases and chaperones are secreted by many pathogenic bacteria [45, 46] and they are involved in processes of colonization and adaptation to stress conditions. In fact, different bacterial species

have shown attenuated virulence, reduced adhesion and biofilm formation when Clp proteins were mutated [47–53].

The complex (GroEL/GroES) is also relevant in terms of virulence, since these proteins can mediate in adhesion and attachment to the host cells [54–56]. Moreover, diverse inhibitors of GroEL/GroES are currently being tested as broad-spectrum antimicrobial agents [57, 58]. Hosogi and Duncan (2005) showed that GroEL and other Hsp mediated the entry of *P. gingivalis* into host epithelial cells [59]. *Porphyromonas gingivalis* GroEL was inhibited by immunization, which significantly reduced levels of alveolar bone loss in experimental animal model [60]. Llama-Palacios *et al.*, in a proteomic study of *A. actinomycetemcomitans* biofilms, also reported increased expression in GroEL, and sera from patients with periodontitis were shown to be immunoreactive against GroEL [61].

Another potential pathogenic mechanism of GroEL proteins is their structural similarity with human Hsp. When overexpressed, GroEL may cause cross-reaction against human Hsp, causing autoantibodies and leading to chronic inflammation [53, 62].

Another important group of genes also found overexpressed in our study were those encoding for proteases, (PG_1060, PGN_1914 and PGN_0952) and peptidases (PG_0088, PGN_2035, PGN_1103, PGN_0788 and PG_1313). These proteases have been related to virulence, since they are able to degrade antibodies and, thus, to evade host tissue defenses and also to be involved in periodontal tissue destruction [63].

When the transcriptomic profile of *P. gingivalis* was studied when growing in a monospecies biofilms [14], only PGN_1914 was up-regulated, which may indicate that the up-regulation of the rest of the described proteases is mainly driven by the presence of other species within the biofilm.

Cell envelope

The present transcriptomic study has revealed differential expression of those genes involved in cell envelope (PG_0679, PG_0922, PG_1039, PG_1180, PG_2224 and PGN_1020). PG_0679 was significantly up-regulated, what is in agreement with previous reports [13]. This gene is associated with antimicrobial resistance in multispecies biofilms, since efflux transporters pump out antimicrobial molecules [64].

However, *P. gingivalis* within a multispecies biofilm showed most of these genes down-regulated, which contrasts with previous reports studying *P. gingivalis* in monospecies biofilms [11, 14]. For example, the putative membrane protein gene PG_1180 and the putative epithelial cell attachment gene PG_2224, were significantly down-regulated.

Although the function is putative or not so concise, in general, the down-regulation of many genes involved in cell envelope biogenesis, taken together, with the down-regulation of metabolism genes involved in energy production or DNA replication, suggest a down-turn in cell replication and a reduced growth rate in biofilm. It has been previously attributed to restricted penetration of nutrients and helps explain the relative resistance of biofilms to antibiotics targeting growth [11].

Genes related with cell envelope lipoproteins, such as PG_0180, PG_0399, PG_1767, PG_1828, PG_2105, PG_2133 and PG_0924, were also differentially expressed. Among them, PG_1828 has been described as a strong cell activator, being major virulence factor for enhancing inflammatory responses [65]. In fact, inhibition of its activity by a deficient mutant suggests a direct link of PG_1828 in the pathogenesis of periodontitis [65].

Quorum sensing

The gene PGN_1827 (*MetK*) demonstrated a significantly higher expression in *P. gingivalis* when growing in a multispecies biofilm. This gene encodes a protein related to the radical S-

adenosyl-l-methionine (SAM) superfamily, the universal signal for quorum sensing (QS). These proteins are responsible of the biosynthetic pathways leading to autoinducer-2 (AI-2) production, which are key in cell-cell communication what affects different bacterial functions related to virulence, such as motility, nutrition, phenotype expressions and modulation, stability and composition of the biofilms [9, 66–69].

Iron metabolism

The present study has shown a significant up-regulation of genes as PGN_0741, a TonB-dependent outer membrane receptor important for iron transportation [70] and *HmuY*. *Hmu* family proteins are important for heme acquisition, which is key for *P. gingivalis*. This bacterium has an absolute growth requirement for heme, which provides them with iron and protoporphyrin IX that cannot be synthesized by itself [41, 71]. *Hmu* family genes has also been implicated as a virulence factor in promoting mononuclear cell-mediated inflammatory responses [72, 73]. The up-regulation of these genes, and in this case *HmuY*, can influence the ability of *P. gingivalis* to promote biofilm formation, as was seen in our previous study, in which the gene *HmuR* was up-regulated when growing in planktonic form in presence of a growing monospecies biofilm [23].

There was also a significant up-regulation of the genes coding for ferritin and ferredoxin (*Ftn*, PGN_0604, PG_1813). Ferritin has been shown to be a requirement for *P. gingivalis* to grow under iron-depleted conditions and peroxide stress [42, 74].

Transposons and CRISPR

P. gingivalis growing within a multispecies biofilm demonstrated up-regulation of several transposons genes (*TraA*, *TraF*, *TraG*, *TraI*, *TraJ*, *TraK*, *TraM*, *TraN*, *TraO*, *TraP*, *TraQ*, PGN_0058, PGN_0056, PG_1061, PGN_0954 and PGN_1912), suggesting that *P. gingivalis*, when growing among competing species, develops horizontal DNA transfer, which may facilitate its adaptation to different micro-environments. This up-regulation has also been reported in other studies [75–77]. Again, these genes were also up-regulated when planktonic *P. gingivalis* was in the presence of a growing biofilm, what may indicate the importance of DNA transfer to allow for survival in different environments and to adapt to an evolving biofilm [23].

Similarly, the genes of the CRISPR system: *Cas2-2* and PGN_1959 were up-regulated in the test group. These genes encode for a CRISPR protein related to CAS2 family, which play broad roles in controlling bacterial pathogenesis, gene regulation and physiology [78], and protect its genome against other surrounding microorganisms and mobile foreign genetic elements, in particular plasmids and transposons [79–81].

Among other functional groups, genes related to ribosome, as *RpsA*, *RpsP*, *RpsT*, *RpmF*, *RpmH*, *RpmL*, *RplQ*, *RplT*, PG_0627 and PGN_0668, were up-regulated in the test group, what presumably indicates increased translation and higher protein synthesis. The fimbria genes *FimA*, *FimC*, *FimD* were down-regulated in *P. gingivalis* within the multispecies biofilm. This fact has also been reported when *P. gingivalis* grew in monospecies biofilm [14], or when planktonic *P. gingivalis* grew in the presence of a monospecies biofilm [23]. Other authors have reported that *FimA* and *Mfa1* were not required for the development of pathogenicity in biofilms [82, 83]. A large proportion of the genes in (S1 Table) of the Supporting Information, demonstrating differential expression when comparing both groups, were however related to proteins of unknown function (40.5%), what indicates the need to further research to understand the functionality of these genes.

This study has clear limitations, such as its *in vitro* nature, the limited number of bacterial species used to develop the biofilms or the lack of influence of the patient's immune response

as well as its physiological condition, which would also influence the gene expression of *P. gingivalis*. Hence, further studies are needed to ascertain the pathogenic capability of *P. gingivalis* in the initiation and progression of periodontitis, as well as the transcriptomic changes that *P. gingivalis* could suffer accompanied by the other species when growing also in planktonic state.

Conclusions

This study has shown that 19.1% of the *P. gingivalis* genome was differentially expressed when it grew within a multispecies biofilm, in comparison with monospecies planktonic growth. Within the biofilm, *P. gingivalis* has shown increased expression of virulence factors and antioxidant enzymes, especially Hsp proteins and several proteases. The identification and quantification of these known genes and other related to unknown proteins may provide new knowledge on the virulence and pathogenicity of this important periodontal pathogen, *P. gingivalis*.

Supporting information

S1 Table. Differentially expressed genes in *Porphyromonas gingivalis* ATCC 33277 in planktonic condition and within a multispecies-biofilm (cutoff ratio $\geq \pm 2$; p-value < 0.05), grouped by functional categories. Microarray data indicate the mean expression fold change and Standard Deviation (SD) of each gene. (DOCX)

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