ORIGINAL ARTICLE

Actinomyces spp. gene expression in root caries lesions

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Background: The studies of the distribution of *Actinomyces* spp. on carious and non-carious root surfaces have not been able to confirm the association of these bacteria with root caries, although they were extensively implicated as a prime suspect in root caries.

Objective: The aim of this study was to observe the gene expression of *Actinomyces* spp. in the microbiota of root surfaces with and without caries.

Design: The oral biofilms from exposed sound root surface (SRS; $n = 10$) and active root caries (RC; $n = 30$) samples were collected. The total bacterial RNA was extracted, and the mRNA was isolated. Samples with low RNA concentration were pooled, yielding a final sample size of SRS = 10 and RC = 9. Complementary DNA (cDNA) libraries were prepared and sequenced on an Illumina® HiSeq 2500 system. Sequence reads were mapped to eight *Actinomyces* genomes. Count data were normalized using DESeq2 to analyse differential gene expression applying the Benjamini-Hochberg correction (false discovery rate [FDR] < 0.001).

Results: *Actinomyces* spp. had similar numbers of reads (Mann-Whitney U-test; $p > 0.05$), except for *Actinomyces* OT178 ($p = 0.001$) and *Actinomyces gerencseriae* ($p = 0.004$), which had higher read counts in the SRS. Genes that code for stress proteins (*clp*, *dna*K, and *gro*EL), enzymes of glycolysis pathways (including enolase and phosphoenolpyruvate carboxykinase), adhesion (Type-2 fimbrial and collagen-binding protein), and cell growth (EF-Tu) were highly – but not differentially ($p > 0.001$) – expressed in both groups. Genes with the most significant upregulation in RC were those coding for hypothetical proteins and uracil DNA glycosylase ($p = 2.61\text{E-}17$). The gene with the most significant upregulation in SRS was a peptide ABC transporter substrate-binding protein (log2FC = −6.00, FDR = 2.37E-05).

Conclusion: There were similar levels of *Actinomyces* gene expression in both sound and carious root biofilms. These bacteria can be commensal in root surface sites but may be cariogenic due to survival mechanisms that allow them to exist in acid environments and to metabolize sugars, saving energy.

Keywords: *RNA-seq*; *Actinomyces spp.*; *root caries*; *transcriptome*; *differential expression*

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Over the past decades, the world's population has been experiencing a higher retention of teeth due to improvements in dental health care. This fact implies an increased number of exposed root surfaces that are susceptible to caries (1). Knowledge of root caries etiopathogeny, and especially the primary etiological factor – the microbial biofilm, can support new research for the development of potentially useful targets for therapeutics. However, the current understanding of biofilm dynamics in root caries remains limited.

The search for a pathogenic species that causes root caries has been the objective of much of the research in this field since the 1970s (2–5). *Actinomyces* spp. are non-acidophilus, gram-positive rods, and facultative anaerobic bacteria that are related to dental plaque aging (6). *Actinomyces viscosus* and *Actinomyces naeslundii* were implicated as the pathogen of root surface caries by animal models and culture-based studies (2, 3, 5, 7). However, studies of the distribution of *Actinomyces* spp. have revealed that biofilm from both sound and carious root

surfaces contains high proportions of *Actinomyces* spp. (8–12). The role of this genus in root caries development or health maintenance may be potentially complex because they could be capable of surviving in both sites, adapting the metabolism to exploit the available substrate.

Many advances in the understanding of dental caries are currently related to the cultured-independent DNA-based methods. The most important advantage would be assessment of the underestimated non-cultivable microorganisms' prevalence in oral biofilms. Studies using *16S rRNA* gene cloning and sequencing confirmed that the predominant bacteria in both carious and sound root surfaces was *Actinomyces* spp., followed by *Streptococcus mutans* and lactobacilli (13). However, DNA-based approaches also have limitations; it is not possible to determine if the DNA is coming from a viable cell, and it is not feasible to determine if the bacteria are expressing virulence factors that contribute to caries development or if they are only present in the environment struggling to survive. Rapid evolution of molecular techniques has enabled understanding not only of the microbiota composition but also of the microbiota function. RNA-based methods have enabled the study of biofilm gene expression, and consequently, the assessment of its functions. Hence, the aim of this study is to compare the gene expression of *Actinomyces* spp. in root surfaces' biofilms with and without caries using the RNA-seq approach.

Materials and methods

Volunteers with an exposed root surface on at least one tooth and no root caries lesions were included in the sound root surface (SRS) group ($n = 10$). Dental biofilms were collected from all available exposed root surfaces. The number of exposed root surfaces varied among individuals. All participants recruited for the root caries (RC) group ($n = 30$) had one primary cavitated root lesion in need of restorative treatment. All lesions showed characteristics of present activity (soft and yellow dentin). Biofilm and carious dentin samples (of soft and infected tissue) were collected from patients during the restorative treatment.

Ethical considerations

This study was approved by the ethics committee of the Federal University of Rio Grande do Sul (process n° 427.168) and by the National Research Ethics Service Committee Yorkshire & the Humber - Leeds West (protocol n° 2012002DD). Volunteers in the study were patients who attended clinics in two centres for any dental treatment: Faculty of Dentistry, Federal University of Rio Grande do Sul, Porto Alegre, Brazil, and Leeds School of Dentistry, University of Leeds, Leeds, UK. All volunteers consented to participate by donating samples after receiving the information about the study.

RNA-seq

After clinical collection, samples were immediately placed in a nuclease-free microtube containing 1 mL of RNA-protect reagent (QIAGEN, Inc., Venlo, Netherlands), transferred to the laboratory, centrifuged at $10,000 \times g$ for 30 s. Pellets were stored at $-80°C$ until further processing.

The total RNA was extracted from all the samples using an UltraClean® Microbial RNA Isolation Kit (Mo-BIO Laboratories, Inc., San Diego, CA) with on-column DNase digestion (QIAGEN, Inc., Venlo, Netherlands). The extracted RNA samples were quantified using the Quant-iT™ RiboGreen® RNA Assay Kit (Invitrogen, Inc., Waltham, Massachusetts, USA), and samples with total RNA concentration < 30 ng/RNA were pooled, leading to a final sample count of 10 SRS and 9 RC (Supplementary Table 1).

A Ribo-Zero™ rRNA Removal Kit (Bacteria), (Epicentre, Illumina, Inc., San Diego, CA) was used for mRNA enrichment, and Illumina® TruSeq™ library prep protocols (Illumina, Inc.) were used for library preparation. Briefly, these steps included: RNA fragmentation, cDNA synthesis, DNA fragment repair, amplification by polymerase chain reaction and purification, and adapter ligation for multiplexing. The final quality of cDNA and library validation was carried out using an Agilent Bioanalyzer (Agilent Technologies). Paired-end sequencing was then performed on an Illumina® HiSeq2500 (Illumina, Inc., San Diego, CA) sequencer to obtain 2×100 bp sequence reads.

Selection of genomes in databases

RNA-seq sequencing data are available from the National Center for Biotechnology Information (NCBI) Sequence Read Archive, under the accession numbers SRS779973 and SRS796739.

Genomes of $n = 162$ bacteria and their associated information were downloaded from the DNA Data Bank of Japan, NCBI, the Broad Institute, and the Human Oral Microbiome Database (HOMD); their data were combined and used as a single organism reference in the short read mapping, which was carried out within the CLC Genomics Workbench 7.5.1 software (CLC Bio, QIAGEN), as described by Do et al. (14). The genomes of eight *Actinomyces* strains were selected for further analysis; these included *Actinomyces gerencseriae* DSM 6844, *Actinomyces johnsonii* F0542, *A. naeslundii* str. Howell 279, *Actinomyces odontolyticus* ATCC 17982, *Actinomyces* sp. oral taxon 170 str. F0386, *Actinomyces* sp. oral taxon 178 str. F0338, *Actinomyces* sp. oral taxon 448 str. F0400, and *Actinomyces oris* (formerly known as *A. viscosus* C505).

Bioinformatics and differential expression

Count tables were generated containing the read count for each oral *Actinomyces* spp. genome. Proportion of genes per

genome expressed (transcripts) was also observed (reads ' > 0' in at least one sample per group). The cut-off for considering the putative presence of the organism in the sample was 2,000 mapped reads (approximately one read per gene).

The relative mean expression (RME) level for *Actinomyces* spp. was calculated for each of the sample groups (SRS and RC) by species. The median of the relative expression values, obtained from the library size estimation carried out within the R package DESeq, was then calculated for each gene. The RME values in all genomes analysed here were added and ranked to observe and compare the most highly expressed *Actinomyces* spp. transcripts in the SRS and RC samples.

Differential gene expression among sample groups was carried out using the R package DESeq2 for each species (15). The cut-off for designating a gene as being differentially expressed was a change in transcript levels of at least 1-log2 fold (a two-fold difference) (positive values = upregulated in RC and downregulated in SRS, negative values = downregulated in RC and upregulated in SRS) and a Benjamini-Hochberg adjusted *P*-value (FDR) of less than 10^{-3} (16). Principal component analysis (PCA) plots were constructed within the DESeq2 package to display the gene expression similarity among samples. The number of overexpressed genes by *Actinomyces* strains was determined per condition (health or disease metabolism).

Results

Eight strains of *Actinomyces* were mapped and evaluated in this study, comprising a total of 21,337 analysed genes. The total number of reads per sample per organism ranged from SRS = 6,250–883,308 and RC = 2,461–2,015,578 (Table 1). Proportion of expressed genes per genome

ranged from 49.3–75.9 (SRS) and 28.0–89.8% (RC). All *Actinomyces* spp. had a similar number of reads and transcripts (Mann-Whitney U-test; *p* >0.05), except for *Actinomyces* OT178 (median of reads = 39,174 in SRS and 4,816 in RC; *p* = 0.001) and *A. gerencseriae* (median of reads = 85,639 in SRS and 47,150 in RC; *p* = 0.004), which had higher read counts for the SRS samples (Table 1). *Actinomyces* sp. OT448 presented the higher number of reads – 883,308 (sample SRS_12) and 2,015,578 (sample RC_H), representing around 60% of total *Actinomyces* gene expression in all samples, RC and SRS, and achieving 86% of total *Actinomyces* gene expression in RC_A (Supplementary Fig. 1). *Actinomyces* spp. OT170 and *A. johnsonii* F0542 had the lowest number of genes per genome expressed in SRS (49.3 and 49.5%), while *Actinomyces* sp. OT178 had the lowest number of genes per genome expressed in RC (only 28% of their total number of genes). The other *Actinomyces* spp. expressed an average of 65% of their genes.

Most expressed genes
The log scale of RME of the 30 highest values at gene level sorted in ascending order for the RC samples are displayed in Fig. 1. Also, the 10 highest values at species level were sorted in ascending order for the RC samples and are displayed in Supplementary Fig. 2. The corresponding values for SRS samples for the same genes were plotted as well. Certain common features could be observed in the expression levels of most genes independent of whether the biofilms came from (RC or SRS).

A subset of genes that code for stress proteins and enzymes of glycolysis pathways was highly, but not differentially (*p* >0.001), expressed in both groups. These genes code for key proteins involved in the maintenance of cell viability and metabolism in oral biofilms, meaning

Table 1. Descriptive analysis of proportion of transcripts and number of significantly upregulated genes (upreg) by species of *Actinomyces* spp.

Strain	Total number of genes	SRS		RC	
		Number of reads Median (range)	Number of upreg[a] (%)	Number of reads Median (range)	Number of upreg[a] (%)
A. gerencseriae DSM 6844	2,875	85,639* (28,147–306,920)	17 (0.6)	47,150 (23,687–385,450)	80 (2.8)
A. johnsonii F0542	3,324	28,930 (8,453–50,070)	21 (0.6)	8,697 (3,020–85,250)	105 (3.2)
A. naeslundii str. Howell 279	2,930	29,657 (8,990–117,608)	19 (0.6)	23,143 (12,438–96,996)	88 (3.0)
A. odontolyticus ATCC 17982	1,982	12,153 (6,250–145,837)	4 (0.2)	5,806 (2,461–55,096)	40 (2.0)
A. sp. oral taxon 170 str. F0386	3,042	25,728 (7,865–53,541)	24 (0.8)	12,577 (3,263–76,652)	89 (2.9)
A. sp. oral taxon 178 str. F0338	2,308	39,174* (15,871–74,3480)	355 (15.4)	4,816 (2,451–43,108)	54 (2.3)
A. sp. oral taxon 448 str. F0400	2,342	45,822 (10,116–883,308)	9 (0.4)	237,720 (11,327–2,015,578)	88 (3.8)
A. oris C505	2,534	58,122 (22,440–619,447)	35 (1.4)	74,763 (22,486–682,971)	226 (8.9)
TOTAL	**21,337**		**484 (2.0)**		**770 (4.0)**

*p <0.05; Mann-Whitney U-test.
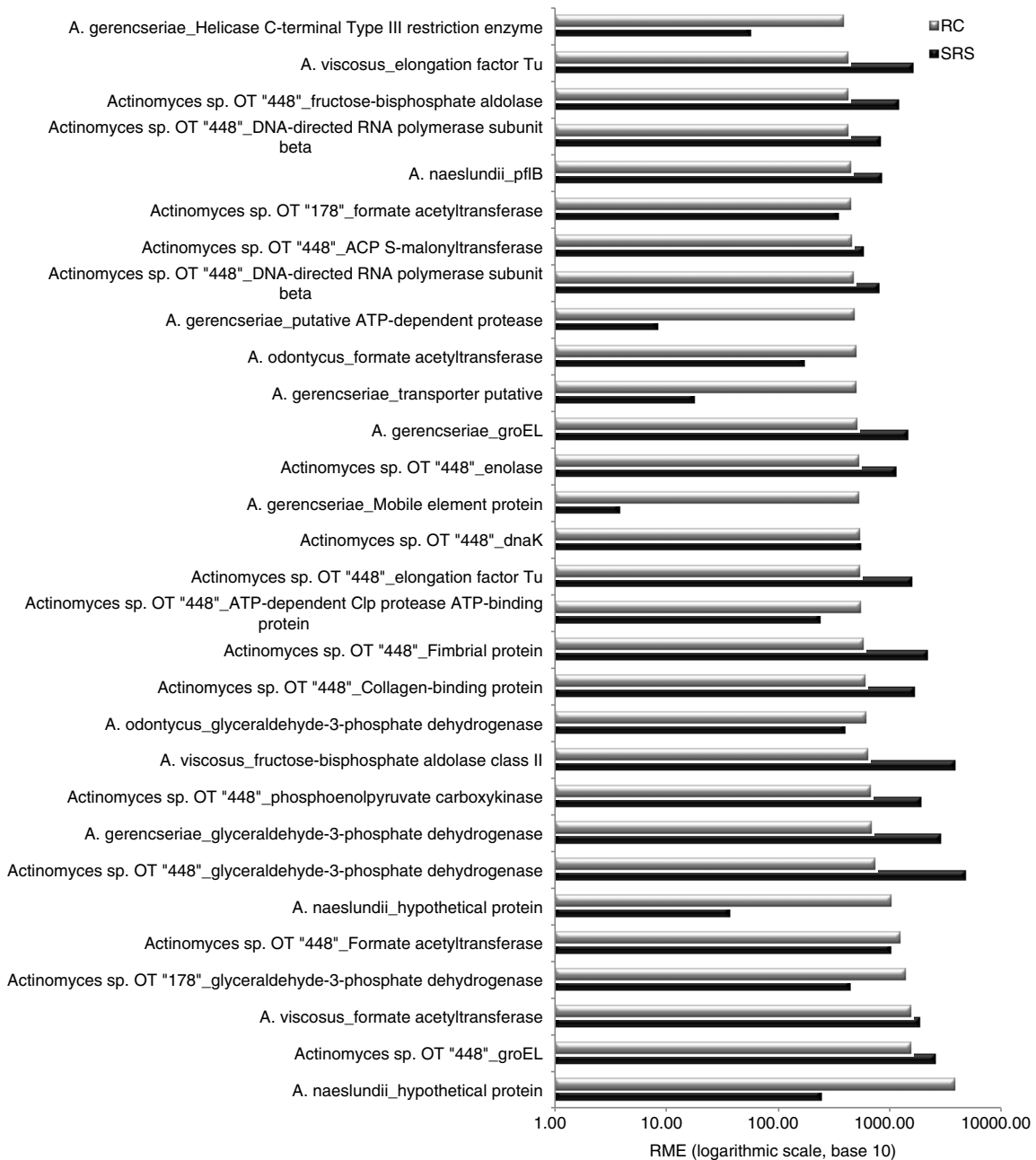[a]Upreg = upregulated genes; FDR <0.001; calculated by DESeq2.

Fig. 1. Relative median expression (RME; log 10) of genes in the sound root surfaces (SRS; $n = 10$) and root caries (RC; $n = 9$) samples. RME was calculated from the median values of normalized read counts. The 30 highest RME values were sorted in ascending order for the RC samples, are displayed with the corresponding values for SRS samples, and indicate genes that are most highly expressed by all *Actinomyces*.

that *Actinomyces* are metabolically active in both caries and sound root surfaces biofilms. Stress-related genes *clp*, *dnaK*, and *groEL* were highly expressed for *Actinomyces* OT448, *A. naeslundii*, and *A. gerencseriae*. The enzymes with functions in glycolysis pathways enolase, formate-acetyltransferase, phosphoenolpyruvate carboxykinase (GTP) (pckA) (gluconeogenesis), and glyceraldehyde-3-phosphate dehydrogenase (*gap*) had higher gene expression for *Actinomyces*. Also, among those highly expressed genes, there were genes coding for proteins related to

adhesion (such as Type-2 fimbrial and collagen-binding protein) and cell growth (EF-Tu).

Differential expression analysis

Using a conservative approach and considering up- or downregulated genes with more than a two-fold difference and FDR $< 10^{-3}$, it was shown that only 5.9% (21,337 genes, 1,254 with significant differential expression) of *Actinomyces* genes had significant differential expression. There were quite a few genes up- and downregulated in

Table 2. Upregulated *Actinomyces* spp. genes in the root caries and in the sound root surfaces samples

		Root caries			Sound root surfaces			
		baseMean	log2FC	FDR		baseMean	log2FC	FDR
A. gerencseriae	Hypothetical protein	4.54	5.98	5.84E-05	LSU ribosomal protein L18p	71.56	−2.78	9.81E-07
	Mobile element protein 14	162.40	4.86	5.07E-10	CRISPR-associated protein Cse4	213.40	−2.66	3.85E-05
	ABC transport protein. ATP-binding subunit	48.37	4.67	5.07E-10	LSU ribosomal protein L9p	46.62	−2.33	4.13E-04
	Hypothetical protein	126.87	4.62	5.14E-09	Aspartate carbamoyltransferase	48.35	−2.30	7.92E-04
	Hypothetical protein	13.85	4.55	1.30E-06	SSU ribosomal protein S19p (S15e)	224.61	−2.30	3.73E-04
	Mobile element protein 13	113.90	4.48	3.39E-11	Glycerol-3-phosphate ABC transporter	599.22	−2.26	5.92E-04
	ATP-dependent DNA helicase	33.01	4.42	1.16E-06	Phosphoenolpyruvate protein phosphotransferase (PTS)	261.52	−2.24	3.04E-04
	Narrowly conserved hypothetical protein	201.96	4.35	5.83E-06	LSU ribosomal protein L4p (L1e)	109.98	−2.23	3.96E-04
	Transcriptional regulator. MerR family	16.91	4.22	5.14E-09	LSU ribosomal protein L5p (L11e)	292.01	−2.18	1.58E-04
	Hypothetical protein	8.95	4.19	5.84E-05	Fructokinase	47.45	−2.17	1.96E-04
A. johnsonii	Hypothetical protein	145.15	6.85	3.61E-15	Cell wall-binding repeat protein	17.08	−4.04	8.35E-04
	Hypothetical protein	4.83	6.67	7.14E-05	L-lactate dehydrogenase	12.05	−3.53	1.97E-04
	Multidrug resistance protein, putative	11.23	6.28	1.42E-06	FIG00545076: hypothetical protein	48.27	−3.28	4.75E-05
	Hypothetical protein	2.69	6.17	2.86E-04	Fructose-bisphosphate aldolase class II	57.29	−3.24	1.88E-08
	5-keto-2-deoxy-D-gluconate-6 phosphate aldolase [form 2]	2.23	6.16	2.14E-04	Hypothetical protein	171.66	−3.13	1.53E-04
	HMPREF1979_01399	2.63	6.14	3.34E-04	Heat shock protein 60 family chaperone GroEL	119.35	−2.97	1.47E-04
	Hypothetical protein	2.77	6.14	3.52E-04	HMPREF1979_01533	14.96	−2.91	3.30E-04
	Predicted L-rhamnose permease RhaY	9.00	6.12	1.62E-05	HMPREF1979_01229	15.90	−2.85	8.37E-05
	Octaprenyl diphosphate synthase/dimethylallyl transferase/(2E,6E)-farnesyl diphosphate synthase/geranylgeranyl diphosphate synthase	94.25	6.11	8.97E-12	HMPREF1979_01249	3.50	−2.84	6.89E-04
	Hypothetical protein	4.50	6.03	4.32E-05	HMPREF1979_01215	5.30	−2.82	9.53E-04
	ABC transporter related	70.12	5.82	1.94E-11	Fructose-bisphosphate aldolase class II	269.02	−3.82	2.69E-10
	Hypothetical protein	53.83	5.69	2.18E-11	Phosphoenolpyruvate carboxykinase [GTP]	17.86	−3.34	1.10E-04
	Sialic acid transporter (permease) NanT	10.33	5.43	5.57E-06	rplN_2_ALJK01000095	38.27	−3.20	1.06E-05
	Inosose dehydratase (EC 4.2.1.44)	12.35	5.26	2.36E-06	SSU ribosomal protein S14p (S29e)	9.53	−3.07	5.14E-04
	Hypothetical protein	2.59	5.18	2.62E-04	NAD-dependent glyceraldehyde-3-phosphate dehydrogenase	287.60	−3.02	1.10E-04
	Hydroxymethylpyrimidine phosphate kinase ThiD (EC 2.7.4.7)	13.71	5.12	2.56E-06	Hypothetical protein	42.89	−2.99	9.03E-04
A. naeslundii	Inosose isomerase (EC 5.3.99.-)	44.85	5.01	4.76E-14	Ferric enterobactin−binding periplasmic protein FepB	17.70	−2.94	2.57E-05

Table 2 (*Continued*)

		Root caries				Sound root surfaces			
		baseMean	log2FC	FDR		baseMean	log2FC	FDR	
	Hypothetical protein	2.49	5.00	2.85E-04	FIG00545076: hypothetical protein	100.64	−2.88	2.37E-07	
	Carotenoid cis-trans isomerase (EC 5.2.-.-)	7.86	4.89	1.10E-04	HMPREF1129_0405_ALJK01000057	30.18	−2.84	3.33E-06	
	Hypothetical protein	2.95	4.84	3.83E-04	L-lactate dehydrogenase	68.53	−2.65	8.82E-05	
	Uracil DNA glycosylase	28.23	6.60	2.61E-17	Hypothetical protein	6.51	−2.92	2.37E-04	
	Para-aminobenzoate synthase	42.91	5.85	3.71E-12	pgk_3_NZ_DS264	74.76	−2.23	2.49E-07	
	Fructose-bisphosphate aldolase	12.63	5.65	4.70E-04	50S ribosomal protein L6	66.95	−1.81	2.18E-04	
	CDP-alcohol phosphatidyltransferase	20.84	5.62	1.07E-08	30S ribosomal protein S8	53.44	−1.79	4.75E-05	
	Membrane protein	1.86	5.55	3.41E-04	DNA helicase	21.23	2.07	4.60E-04	
A. odontolyticus	LysR family transcriptional regulator	1.72	5.34	6.94E-04	Sodium:proton antiporter	21.21	2.58	3.32E-04	
	MFS transporter	19.96	5.32	1.34E-08	Lipoate−protein ligase A	9.86	2.59	5.03E-04	
	Two-component system response regulator	3.69	4.99	2.17E-04	Uracil−xanthine permease	60.81	2.69	2.18E-04	
	Hypothetical protein	2.51	4.95	7.01E-04	ABC transporter ATP-binding protein	2.82	2.95	3.16E-04	
	Glycosyltransferase family 1	2.00	4.87	5.03E-04	Sialic acid transporter	3.80	2.99	6.27E-04	
	Transcriptional regulator, AraC family	5.41	6.58	5.10E-05	Fructose-bisphosphate aldolase class II	133.34	−4.27	5.68E-10	
	HMPREF9056_00703_AFBL01000016	2.42	5.86	5.17E-04	Ferric enterobactin-binding periplasmic protein FepB	10.32	−3.89	1.22E-04	
	Probable transposase for insertion sequence	33.82	5.82	2.50E-09	SSU ribosomal protein S14p (S29e)	5.46	−3.60	9.23E-04	
	L-xylulose 5-phosphate 3-epimerase	12.25	5.78	4.33E-06	Sucrose-6-phosphate hydrolase	20.86	−3.22	1.94E-04	
Actinomyces	HMPREF9056_00704_AFBL01000016	2.18	5.70	8.18E-04	LSU ribosomal protein L6p (L9e)	26.16	−3.22	9.65E-05	
OT170	Hypothetical protein	2.05	5.65	9.47E-04	FIG00545076: hypothetical protein	108.49	−3.01	1.69E-09	
	Hypothetical protein	7.31	5.58	3.01E-05	Multiple sugar ABC transporter, membrane-spanning permease protein MsmF	47.15	−2.98	5.71E-05	
	FIG00448805: hypothetical protein	8.66	5.55	3.34E-06	Glycerol-3-phosphate ABC transporter, ATP-binding protein UgpC (TC 3.A.1.1.3)	202.36	−2.91	2.44E-05	
	Hypothetical protein	35.13	5.49	2.46E-07	Pyruvate formate-lyase (EC 2.3.1.54)	23.67	−2.90	5.97E-04	
	Putative ABC transporter ATP-binding protein	16.21	5.49	5.82E-06	SSU ribosomal protein S7p (S5e)	12.59	−2.88	6.09E-06	
	Hypothetical protein	4.04	5.53	1.36E-04	Peptide ABC transporter substrate-binding protein	4.82	−6.00	2.37E-05	
	Polysaccharide biosynthesis protein	26.83	5.31	7.22E-12	Hypothetical protein, partial	3.99	−5.89	3.51E-05	
	Inosine-uridine nucleoside N-ribohydrolase	7.84	4.88	2.36E-05	Co-chaperone GrpE, partial	4.16	−5.80	5.74E-05	
	ATP-dependent Lon protease	20.64	4.61	2.25E-06	Lipase	3.45	−5.64	9.37E-05	
Actinomyces	Plasmid replication-like protein	3.02	4.56	6.84E-04	Membrane protein	4.34	−5.59	1.59E-04	
OT178	Molybdopterin biosynthesis protein MoeB	5.70	4.43	1.33E-05	glmZ(sRNA)-inactivating NTPase	3.11	−5.51	1.51E-04	

Table 2 (*Continued*)

		Root caries				Sound root surfaces		
		baseMean	log2FC	FDR		baseMean	log2FC	FDR
	CDP-alcohol phosphatidyltransferase	13.53	4.42	1.91E-10	Hypothetical protein	3.05	−5.50	1.59E-04
	Two-component system response regulator	13.77	4.39	1.10E-14	Thioredoxin	3.02	−5.43	2.04E-04
	Hypothetical protein	12.16	4.36	4.31E-06	Sugar-binding protein	6.15	−5.40	2.98E-06
	50S ribosomal protein L33	9.87	4.30	1.32E-13	Dihydroxyacetone kinase	3.04	−5.36	3.04E-04
Actinomyces OT448	MFS transporter	25.71	5.20	8.72E-06	ABC transporter substrate-binding protein	998.79	−2.47	9.57E-05
	Hypothetical protein	2.88	5.02	6.14E-04	Elongation factor Ts	365.12	−2.27	1.25E-04
	Transposase	5.77	4.86	2.24E-04	50S ribosomal protein L23	314.22	−2.14	5.23E-04
	Hypothetical protein	2.53	4.79	2.63E-04	HMPREF9062_RS03960	40.36	−1.89	9.57E-05
	Hypothetical protein	2.42	4.76	6.85E-04	30S ribosomal protein S3	384.20	−1.88	1.58E-04
	Major facilitator superfamily protein	3.40	4.63	4.97E-04	50S ribosomal protein L2	385.80	−1.75	8.83E-05
	Fumarate hydrolyase	6.90	4.15	7.19E-05	F0F1 ATP synthase subunit gamma	97.09	−1.56	1.90E-05
	Type II secretion system protein	17.26	4.08	9.28E-07	50S ribosomal protein L22	173.31	−1.51	8.43E-04
	Hypothetical protein	37.88	4.03	1.07E-06	50S ribosomal protein L1	129.96	−1.33	9.77E-04
	Hypothetical protein	3.63	3.95	8.10E-04	Hypothetical protein HMPREF9062_RS10410	37.18	1.36	9.20E-04
A. oris C505	Hypothetical protein	3.88	5.78	7.68E-06	CRISPR-associated protein Cse1	5.17	−3.94	3.34E-05
	XRE family transcriptional regulator	9.19	5.10	5.29E-07	groEL	1441.54	−2.76	7.42E-05
	Hypothetical protein	3.79	4.65	2.52E-05	Fructose-bisphosphate aldolase class II	4512.01	−2.68	3.68E-04
	CAAX amino protease	6.94	4.52	2.82E-06	1,4-alpha-glucan-branching protein	463.60	−2.67	2.82E-06
	Hypothetical protein	1.27	4.52	7.48E-04	50S ribosomal protein L9	74.66	−2.51	5.23E-07
	Cro/CI family transcriptional regulator	3.05	4.36	3.96E-04	Universal stress protein	18.39	−2.51	6.15E-04
	ABC transporter ATP-binding protein	1.93	4.33	7.83E-04	Sugar transporter	36.13	−2.50	5.29E-07
	HMPREF0059_RS05590	3.14	4.26	4.21E-05	30S ribosomal protein S11	126.46	−2.39	9.65E-05
	Aminoglycoside phosphotransferase	3.18	4.24	5.54E-05	Elongation factor Ts	197.32	−2.34	2.17E-05
	WXG100 family type VII secretion target	8.53	4.23	3.86E-06	Sugar ABC transporter permease	198.18	−2.30	2.50E-04

The list of genes is ranked in descending order to the log2FoldChange values. Positive log2FoldChange represents the upregulated genes in RC (or downregulated in SRS); Negative log2FoldChange represents upregulated genes in SRS (or downregulated in RC).

baseMean = the average of the normalized count values (divided by size factors).

log2FC, log2FoldChange: corresponds to the effect size estimate indicating the change in gene expression between both sample groups; FDR, false discovery rate; Benjamini and Hochberg adjusted *p*-values.

the samples independent of whether they belonged to a sound or a caries biofilm. RC presented a higher number of overexpressed genes ($n = 484$ genes in SRS and $n = 770$ genes in RC) (Table 1). *A. oris* C505 had the highest differential expression (8.9% of genes upregulated in RC). Only *Actinomyces* OT178 presented a large number of

overexpressed genes in SRS (355 upregulated and 54 downregulated genes in SRS).

Table 2 displays the most significantly differentially expressed genes for each condition. The bioinformatics tool DESeq2 suggested that genes with the most significant upregulation in RC were those coding for many
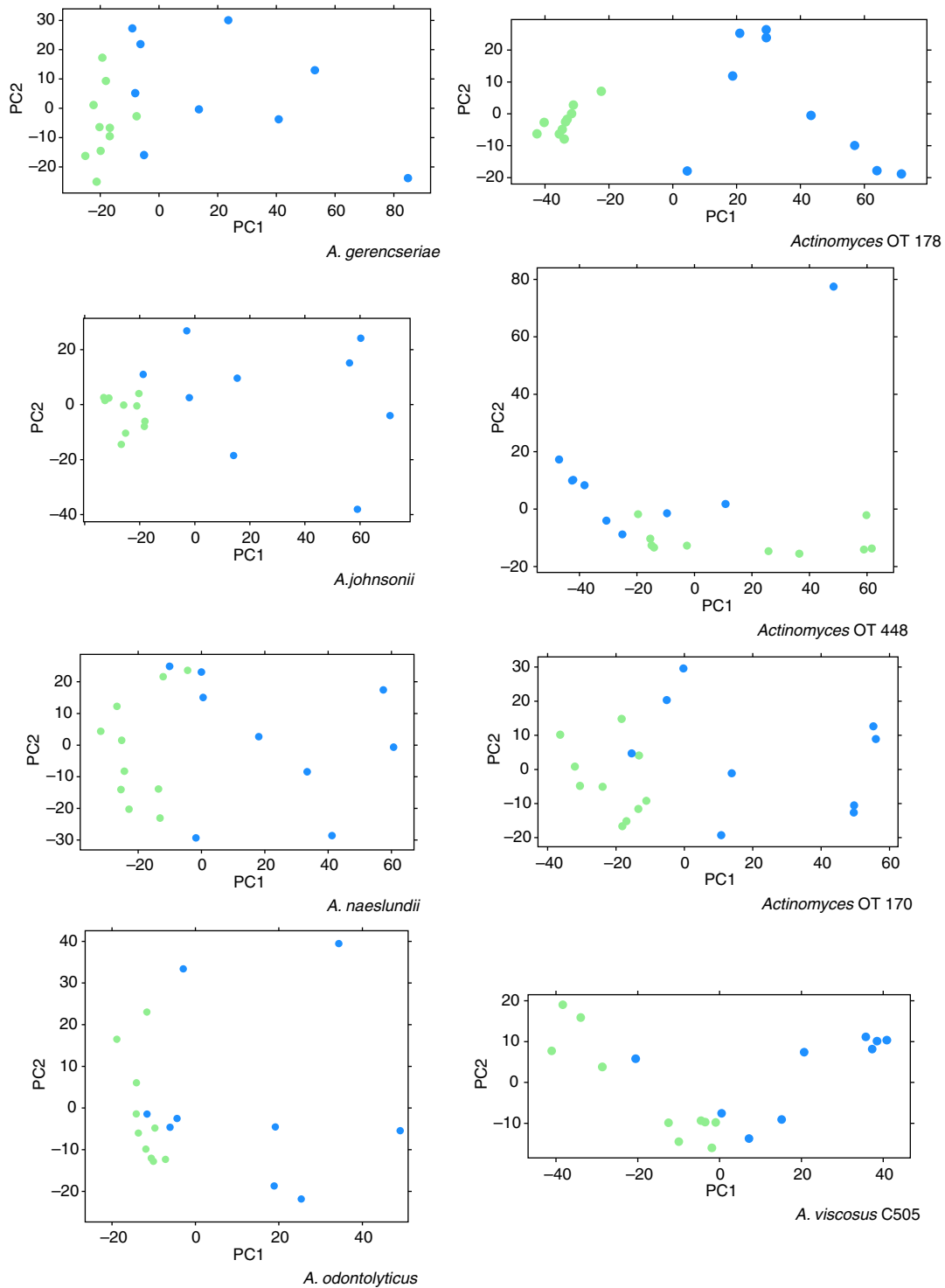
Fig. 2. Principal component analysis (PCA) plots displaying sample-to-sample distances for root caries' (blue) and sound root surfaces' (green) biofilms based on the differential expression by *Actinomyces* spp.

hypothetical proteins and uracil DNA glycosylase (log2FC = 6.60; FDR = 2.61E-17), which is an important protein for transcription and mutagenesis prevention.

Two systems responsible for alkalinization of the biofilm had upregulated genes in RC: arginine biosynthesis and urea catabolism. In arginine biosynthesis, some genes had significant upregulation in RC (none upregulated in SRS). *A. oris* C505 was coding for arginine deiminase (log2FC = 3.2, FDR = 0.00003); *Actinomyces* OT178 was coding for arginine ABC transporter ATP-binding protein (log2FC = 3.77; FDR = 2.09E-10); and *A. oris* C505 was coding for arginine ABC transporter ATP-binding protein (log2FC = 1.75; FDR = 0.0009). In the urea metabolism, one gene showed significant upregulation in RC: urea transporter (log2FC = 2.8; FDR = 0.0006) expressed by *A. oris* C505.

In the case of significant downregulated genes in RC, the most significant one was a peptide ABC transporter substrate-binding protein (log2FC = −6.00, FDR = 2.37E-05). Several hypothetical proteins were identified as well as proteins such as lipase (log2FC = −5.64, FDR = 9.37E-05), membrane proteins (log2FC = −5.59, FDR = 1.59E-04), glycosyl transferase family 2 (log2FC = −5.22, FDR = 4.15E-04), amino acid and carbohydrate ABC transporters (log2FC = −5.14, FDR = 2.99E-13), and others. The enzyme lactate dehydrogenase was upregu-

lated in SRS (none in RC), expressed by *A. oris* C505 (log2FC = −2.02), *A. gerencseriae* (log2FC = −1.82), *A. johnsonii* (log2FC = −3.53, FDR = 7.30E-06), *Actinomyces* OT178 (log2FC = −3.08, FDR = 3.06E-10), and *A. naeslundii* (log2FC = −2.65, FDR = 1.73E-06).

Figure 2 shows PCA plots that were based on the transcriptomic data mapped to each *Actinomyces* strain. It indicates larger differences among RC samples than among SRS samples, suggesting that metabolic functions in the caries lesions are less conserved than in the SRS samples.

Metabolic pathways

Figure 3 shows the number of overexpressed genes per group (SRS and RC). A higher number of genes involved in amino acid metabolism were overexpressed in RC samples. Nucleotide metabolism had a higher number of overexpressed genes in SRS. Both groups of samples (SRS and RC) indicated overexpression of genes associated with carbohydrate metabolism.

Figure 4 shows the starch and sucrose metabolism pathways, highlighting the overexpressed functions in SRS and RC. As can be observed, all overexpressed genes are different among groups. While in RC, *Actinomyces* spp. especially seem to be metabolizing simple sugars (as sucrose and galactose), in SRS they are metabolizing
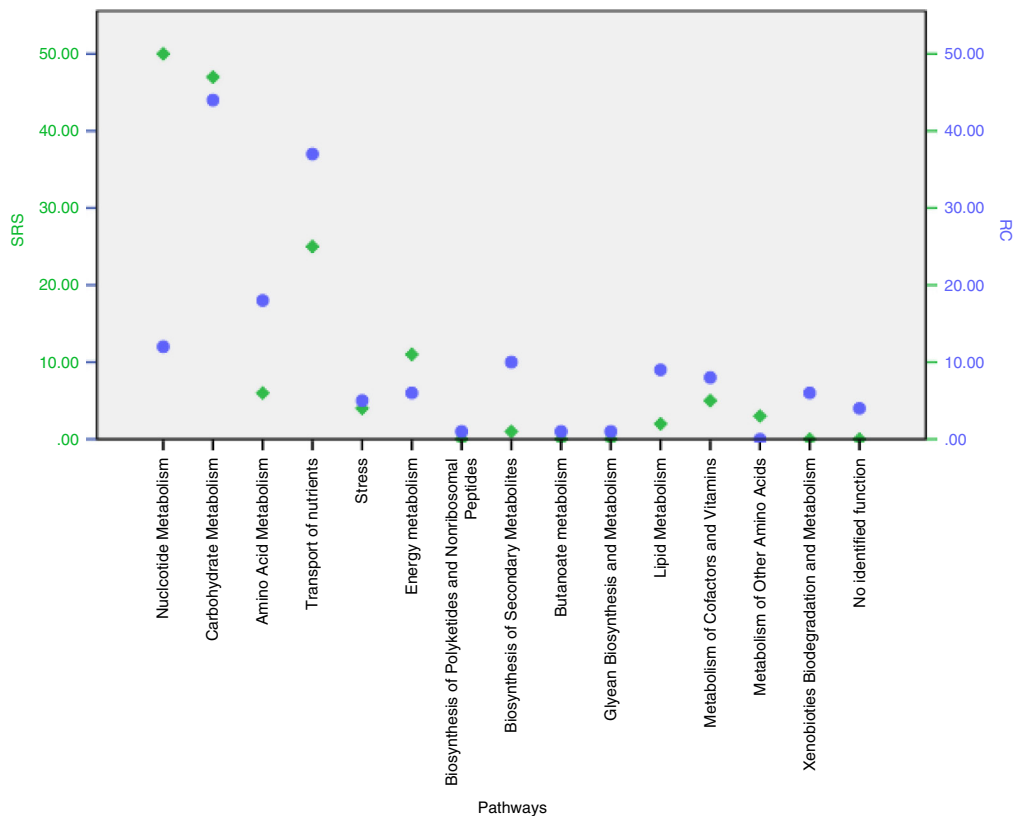

Fig. 3. Number of overexpressed genes by pathway class for root caries' (blue) and sound root surfaces' (green) biofilms based on the differential expression analysis for the selected *Actinomyces* spp.

a)

b)

Fig. 4. Continued

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glycogen. For example, the enzyme glycogen phosphorylase, which catalyses the first step in the degradation of large branched glycan polymers, as starch, was over-expressed in SRS, coded by *Actinomyces* OT178, *Actinomyces* OT170, *A. oris* C505, *A. naeslundii*, and *A. gerencseriae*. *A. naeslundii* was overexpressing sucrose phosphorylase in RC and glycogen phosphorylase in SRS. *A. johnsonii* was overexpressing alpha-glucosidase, which has a function in galactose metabolism in RC and 4-alpha-glucanotransferase (amylomaltase) in SRS. *Actinomyces* OT170 was also overexpressing alpha-glucosidase and sucrose phosphorylase in RC; although in SRS, this species was overexpressing PTS system, beta-glucoside, sucrose-6-phosphate hydrolase, and glycogen phosphorylase. *Actinomyces* OT178 was overexpressing Alpha-amylase and Alpha-1,4-glucan–maltose-1-phosphate maltosyl-transferase in RC; and in SRS, it was overexpressing fructokinase, UTP–glucose-1-phosphate uridylyltransferase, glycogen phosphorylase, and alpha-1,6-glucosidase. *A. gerencseriae* was overexpressing fructokinase in SRS, and *A. oris* C505 was overexpressing glucose-6-phosphate isomerase, glycogen phosphorylase, and 1,4-alpha-glucan-branching in SRS. The arginin metabolism pathway can be consulted in Supplementary Fig. 3.

Discussion

Many studies have investigated the microbiota associated with human tooth root surfaces in health and disease by culture-based approaches, with the aim of detecting the root caries pathogen (7, 9, 11–13, 17–22). It was believed that a unique microbiota from root surfaces with and without caries lesions would be comprised mainly of *Actinomyces* spp. The present analysis focused on transcriptional dynamics among eight abundant *Actinomyces* spp. in oral microbial populations. Overall, exposed root surface samples were collected from larger surface areas compared to the lesions, which could affect differential analyses; however, normalizations steps were applied, and results showed consistently high levels of gene expression in all *Actinomyces* strains in both sample groups (RC and SRS). Additionally, the very low number of differentially expressed genes (5.9%) indicates that the *Actinomyces* present have very similar metabolisms in both caries and caries-free sites. This means that *Actinomyces* spp. seem able to easily exploit this niche, and that they are able to colonize and proliferate in and on root surfaces in a healthy and cariogenic environment.

The analysed species of *Actinomyces* seem to have a small number of genes with significantly higher expression for putative cariogenic functions, except for *Actinomyces* OT178, which had some overrepresented functions in health-related biofilm. The present study found a high expression level of some genes related to cell wall metabolism (Fig. 1) in health biofilms and lesions. Other studies that used RNA-seq to identify the transcriptome/metatranscriptome of oral biofilms also found overrepresentation of elongation factors Tu and G, underlining their involvement in oral biofilm formation (14, 23, 24). Likewise, putative transposase and multidrug resistance protein were upregulated in RC. These genes take part in mechanisms that can transfer genes or plasmids from a pathogen to oral commensal bacteria. In bacteria, transposons can contain genes for antibiotic resistance or can modify the phenotype of other species by mutation, which can be related to the higher potential for genetic mobility when growing as a biofilm. In another study that evaluated the metatranscriptome of biofilms related to periodontal disease, transposases were overexpressed by *Lactobacillus casei* and *Streptococcus mitis* in pathogenic biofilms (25). Furthermore, because many hypothe-

Fig. 4. Starch and sucrose metabolism pathway obtained from Kyoto Encyclopedia of Genes and Genomes (KEGG) and displaying overexpressed genes (pink boxes) for root caries and sound root surfaces biofilms, based on the differential expression analysis of selected *Actinomyces* spp.
Overexpressed in RC: Maltodextrin glucosidase (EC 3.2.1.20) expressed by *A. johnsonii* (Log2FC = 3.47; FDR = 4.54E-05) and by *Actinomyces* OT170 (Log2FC = 3.71; FDR = 1.53E-05). Sucrose phosphorylase (EC 2.4.1.7) expressed by *Actinomyces* OT170 (Log2FC = 2.32; FDR = 1.56E-05) and by *A. naeslundii* (Log2FC = 2.51; FDR = 1.85E-04). Alpha-amylase (EC 3.2.1.1) expressed by *Actinomyces* OT178 (Log2FC = 3.08; FDR = 5.95E-07).
Overexpressed in SRS: PTS system, beta-glucoside-specific IIB component (EC 2.7.1.69) expressed by *Actinomyces* OT170 (Log2FC = −2.57; FDR = 4.17E-08). Sucrose-6-phosphate hydrolase (EC 3.2.1.26) expressed by *Actinomyces* OT170 (Log2FC = −3.22; FDR = 1.94E-04). Fructokinase (EC 2.7.1.4) expressed by *Actinomyces* OT178 (Log2FC = −3.07; FDR = 1.10E-05) and by *A. gerencseriae* (Log2FC = −2.17; FDR = 1.96E-04). UTPglucose-1-phosphate uridylyltransferase (EC 2.7.7.9) expressed by Actinomyces OT178 (Log2FC2.34; 1.83E-04). Glucose-6-phosphate isomerase (EC 5.3.1.9) expressed by *A. oris* C505 (Log2FC = −1.97; FDR = 6.53E-05). Glycogen phosphorylase (EC 2.4.1.1) expressed by *A. gerencseriae* (Log2FC = −2.06; 3.62E-04), *Actinomyces* OT178 (Log2FC = −1.98; FDR = 3.62E-04), *A. oris* C505 (Log2FC = −1.92, FDR = 4.07E-04), *A. naeslundii* (Log2FC = −1.79; FDR = 5.79E-04), and *Actinomyces* OT170 (Log2FC = −1.72, FDR = 6.42E-05). 1,4-alpha-glucan-branching (EC 2.4.1.18) protein expressed by *A. oris* C505 (Log2FC = −2.67; FDR = 2.82E-06). Alpha-1,6-glucosidase expressed by *Actinomyces* OT178 (Log2FC = −3.28; FDR = 2.26E-06) (EC 3.2.1.33). 4-alpha-glucanotransferase (amylomaltase) (EC 2.4.1.25) expressed by *A. johnsonii* (Log2FC = −2.91; FDR = 3.30E-04). Alpha-1,4-glucan--maltose-1-phosphate maltosyltransferase (EC 2.4.99.16) expressed by *Actinomyces* OT178 (Log2FC = −2.20; FDR = 8.72E-07).

tical proteins were upregulated in both conditions, their functions should be studied to clarify the relationship of *Actinomyces* to the development of root caries, even though *Actinomyces* previously have been described as the most prevalent bacteria in root surfaces (2, 7, 12, 13, 19, 26).

It was suggested that microorganisms involved in root caries are less dependent on refined carbohydrates than in coronal caries (9). In the present study, many genes related to glycolysis/gluconeogenesis pathways were identified, concluding that *Actinomyces* generate energy mainly from glycolysis in both healthy and carious root sites but using different enzymes (Figs. 3 and 4). This suggests the importance of sugar availability for the genus' prevalence in the plaque, despite reports of their low saccharolytic activities (27). Likewise, this also suggests that the metabolism of carbohydrates is the most altered function of *Actinomyces* in a biofilm in homeostasis and dysbiosis. *Actinomyces* cells metabolize carbohydrates to organic acids and can also accumulate intracellular polysaccharides, which represent a cariogenic trait in these bacteria.

The main route to degrade glucose is the Embden-Meyerhof-Parnas (EMP) pathway, which is widely distributed in saccharolytic bacteria. However, it was already shown that *A. naeslundii* cells operate in this pathway in a manner different from that of the oral streptococci, which comprise the other dominant part of the saccharolytic bacteria in dental plaque and whose glycolysis has been studied intensively (28). *Actinomyces* use the compounds of polyphosphate and phosphoenolpyruvate carboxykinase (GTP) (also called *pckA* in *Actinomyces* OT448) as phosphate donors for hexokinase synthesis and phofructokinase phosphatase instead of ATP. GTP converts oxaloacetate into phosphoenolpyruvate and carbon dioxide. Further metabolism will generate the end products lactate, formate, acetate, and succinate through the regular EMP pathway (27, 28) like most other saccharolytic bacteria. These specific initial steps of phosphorylation could be implicated in the pathogenesis because this mechanism gives some advantage to *Actinomyces* cells for energy saving. In the results of this study, phosphoenolpyruvate carboxykinase was one of the higher expressed genes in RC and SRS, with no differential expression (very similar gene expression in both conditions).

Adhesion of bacteria to tooth surfaces is an essential step in the initiation of bacterial pathogenesis. It is already known that *Actinomyces* spp. can colonize the cervical surfaces and the gingival margin, contributing also to periodontal disease (3, 4). Numerous adhesion factors have been studied and implicated in the virulence of these bacteria, especially for periodontal diseases. Bacteria express a diverse array of fimbriae that are involved in bacterial adherence (29) and interbacterial adhesive interactions (30). Fimbriae type 1 and type 2 may confer

some advantage in the establishment of *Actinomyces* (29). Different fimbriae-binding properties may explain the presence of *Actinomyces* in root sites. High expression of genes that code Type-2 fimbriae were found in both conditions, which relates to epithelial adhesion. Perhaps this confers advantages for *Actinomyces* adhesion in root surfaces (relationship with the gingival tissue) and also for the community biofilm co-adhesion. Also, collagen-binding protein was highly expressed and could be involved in the *Actinomyces* adhesion to root sites. It was previously demonstrated that the strains of *Actinomyces* that possess fimbriae exhibited strong binding to collagen. Therefore, the avidity of *Actinomyces* spp. for collagen would seem to be at least partially responsible for the high proportions of these organisms found on root surfaces sites (31).

The role of this genus in root caries development or health maintenance may be potentially complex because its species could be capable of surviving in both sites by being able to adapt the metabolism and utilize the available substrate. Several stress-related genes were highly expressed in RC, which means that these species developed mechanisms to survive in that inhospitable environment. One of these mechanisms was the use of lactate as a carbon source. The acidic environment within carious lesions is unlikely to be homogenous despite lactic acid being the major organic acid present (23). Lactate metabolism is an important pH regulating mechanism that facilitates the pH neutralization-producing propionate, acetate, CO_2, and H_2. Bacteria that utilize organic acids as their energy source may contribute to maintaining health by serving as an acid sink (32). Lactate could be converted to pyruvate by NAD-independent lactate dehydrogenase and further to acetyl CoA by pyruvate dehydrogenase. It was demonstrated that *A. naeslundii* strains degrade lactate, aerobically, to acetate and CO_2 through the conversion of lactate into pyruvate by a NAD-independent lactate dehydrogenase (28). The results of this study showed that the lactate dehydrogenase was upregulated in SRS in *Actinomyces* OT178, *A. naeslundii*, *A. johnsonii, A. gerencseriae*, and *A. oris* C505. This mechanism may have implications for *Actinomyces'* ability to preserve the pH homeostasis in root surfaces' biofilm.

In conclusion, the results of the present study showed that *Actinomyces* metabolism is very high and very similar in both health and caries root surface samples. *Actinomyces* OT178 seems to have some specific functions in health-related biofilms. As determined by past evidence, which found a high prevalence of *Actinomyces* in both sound and root caries sites, these results indicate their presence as commensals but do not preclude them from contributing to cariogenicity through the expression of fimbriae components for adhesion, genetic mobility, and energy saving for sugar metabolism. However, it is

important to point out that these functions in biofilms may be compensated by other non-*Actinomyces* species, and thorough gene expression analysis of the whole microbial community could help explain these interactions.

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Conflict of interest and funding

The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

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