Gene expression profile of *Scardovia* spp. in the metatranscriptome of root caries

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Abstract: A few investigations of caries biofilms have identified *Scardovia* spp.; however, little is known about its involvement in caries pathogenesis. The purpose of this study was to assess the gene expression profile of *Scardovia* spp. in root caries, and compare it with other microorganisms. Clinical samples from active root caries lesions were collected. Microbial mRNA was isolated and cDNA sequenced. The function and composition of the Scardovia were investigated using two methods: a) *de novo* assembly of the read data and mapping to contigs, and b) reads mapping to reference genomes. Pearson correlation was performed ($p < 0.05$). Proportion of *Scardovia inopinata* and *Scardovia wiggsiae* sequences ranged from 0-6% in the root caries metatranscriptome. There was a positive correlation between the transcriptome of *Lactobacillus* spp. and *Scardovia* spp. ($r = 0.70$; $p = 0.03$), as well as with other Bifidobacteriaceae ($r = 0.91$; $p = 0.0006$). Genes that code for fructose 6-phosphate phosphoketolase (the key enzyme for "Bifid shunt"), as well as ABC transporters and glycosyl-hydrolases were highly expressed. In conclusion, "Bifid shunt" and starch metabolism are involved in carbohydrate metabolism of *S. inopinata* and *S. wiggsiae* in root caries. There is a positive correlation between the metabolism abundance of *Lactobacillus* spp., Bifidobacteriaceae members, and *Scardovia* in root caries.

Keywords: Root Caries; High-Throughput Nucleotide Sequencing; RNA; Biofilms.

Introduction

Dental biofilms are microbial communities with a complex structure, in which each organism has specific requirements for growth and survival. Consequently, many species, metabolic pathways for utilisation of different nutrients and cell protection against stress factors are found in a biofilm.[1] This diversity of species and metabolism conserves the homeostasis within the biofilm,[2] which can be disrupted by an environmental change that leads to progression to caries.[3] Many species have been found in dental caries biofilms,[4] but studies still focus on well-established cariogenic microorganisms, *Streptococcus mutans* and *Lactobacillus* spp. *S. mutans* is well-documented as an important (but not necessary) organism for caries. *Lactobacillus* spp. are associated with root caries progression, but were absent or rarely observed in individuals without root caries,[5]

in accordance with the concept that they are weakly adherent and depend on ideal niches with retention to be able to colonize.[6]

Some studies have unveiled a massive prevalence of the Bifidobacteriaceae family in both coronal[7,8] and root caries.[9,10] Similarly to lactobacilli, the Bifidobacteriaceae family has no ability to form biofilms by itself,[11] requiring a retentive niche to colonize. Some species of this family have been consistently found in root caries lesions, and amongst them, *Scardovia* spp. has been identified as a member of caries lesion biofilms, especially in severe early childhood caries.[12,13,14] *Scardovia* spp. was one of the members of the oral microbiota with low abundance (< 0.01%) that significantly influenced the microbial community structure and could be associated with disruption of homeostasis.[15] Prevalence of this genus was associated with root caries activity.[9,10,16]

It is still unknown whether *Scardovia* spp. metabolism may play a major role in dental caries biofilms. Their cariogenic potential was investigated in a few studies[11,17] and their acid production was shown to be higher when in association with *S. mutans in vitro*.[11] As heterofermentative organisms, *Scardovia* spp. can metabolize several carbohydrates with lactate and acetate as major end products of fermentation.[18] These products could help in the biofilm acidification leading to dysbiosis. The purpose of this study was to explore the gene expression profiles of *Scardovia inopinata* and *Scardovia wiggsiae* in root caries, and compare it with that of *Streptococcus mutans, Lactobacillus* spp., which are well-established cariogenic microorganisms, and other Bifidobacteraceae members.

Methodology

Clinical collection and experimental approach

Volunteers to the study were patients who attended the dental clinics for any dental treatment in two centres: a) Faculty of Dentistry, Federal University of Rio Grande do Sul, Porto Alegre, Brazil; and b) Leeds School of Dentistry, University of Leeds, Leeds, United Kingdom. This study was approved by the ethics committee of the Federal University of Rio Grande do Sul (process no 427.168) and by the ethics

committee of the NRES Committee Yorkshire & The Humber - Leeds West (protocol nº 2012002DD). Ethical approval was obtained for the samples collection and all volunteers signed a formal consent.

Details of the clinical collection and experimental approach are described elsewhere.[19] Briefly, clinical samples (biofilm + carious dentin) were collected from volunteers (n = 30) presenting an active cavitated root caries lesion. The diagnosis of root caries was done according to the activity criteria, using visual-tactile examination.[20] Excavated carious dentin containing soft and infected tissue was collected using a sterile spoon excavator (SSWhiteDuflex, Rio de Janeiro, Brazil). The mean age of the volunteers who donated root caries samples was 60.1 ± 11.6 years (range 40–90 years).

After collection, samples were immediately placed in a reagent for RNA stabilization (Qiagen Inc.. Manchester, UK). The total RNA was extracted using the UltraClean® Microbial RNA Isolation kit (Mo-bio, San Diego, USA) with on-column DNAse digestion (Qiagen Inc., Manchester, UK). Samples with a total RNA concentration < 30ng/RNA were pooled, leading to a final sample of seven pools and two unique samples, with good mRNA quantity and quality. The total RNA was depleted of ribosomal RNA using the Ribo-Zero™ Meta-Bacteria Kit (Epicentre, Illumina, San Diego, USA). Illumina®TruSeq™ library prep protocols (Illumina, San Diego, USA) were used for library preparation from the enriched mRNA. Sequencing was performed on the Illumina HiSeq2500 (Illumina Inc., San Diego, USA) sequencer to obtain 2 x 100bp sequence reads.

Bioinformatics analysis

RNA sequencing data are available from the National Center for Biotechnology Information (NCBI) Sequence Read Archive, under the accession number SRS779973. Two different pipelines were used to carry out the bioinformatics analysis, as described below.

Pipeline a) Annotation of de novo assembled contigs

Read data were obtained as FASTQ files and were quality trimmed using *cutadapt* (https://

github.com/marcelm/cutadapt). The taxonomic profiling was carried out by *denovo* assembly of the reads into contigs using the default parameters at CLC Genomics Workbench 7.5.1 software (CLC Bio, Qiagen, Manchester, UK). Species and gene annotation of the contigs was carried out using *diamond* (https://ab.inf.uni-tuebingen.de/software/diamond/) with the NCBI nr protein database (downloaded in December 2017), with settings of 70% similarity to the reference database. Reads from each sample were then mapped against the contigs using the CLC Genomics Workbench 7.5.1 software and a read count table was obtained.

The number of reads assigned to *Scardovia* spp., Bifidobacteriaceae members, *Lactobacillus* spp. and *S. mutans* in the total metatranscriptome was assessed and plotted (RStudio for Mac, version 1.1.463, Plotly R package, ggplot2 R package). The proportion of species in the total metatranscriptome was calculated as follows: [*sum of unique reads by species or genera / total number of mapped reads *100*]. This was considered as the relative abundance of each species/genus in each sample. Pearson correlation was performed to evaluate the degree of correlation (and the direction of correlation - whether positive or negative) between the appraised organisms (RStudio, base R package; $p < 0.05$), after checking the normality of the data with the Shapiro-Wilk test.

Pipeline b) Read mapping to 162 oral bacterial genomes

Scardovia spp. gene expression was analysed by mapping reads to 162 oral bacterial reference genomes, including *S. inopinata* JCM 12537 and *S. wiggsiae* F0424. Quality-trimmed FASTQ files for each sample were imported into the CLC Genomics Workbench 7.5.1 software (CLC Bio, Qiagen, Manchester, UK). Genomes and their associated information were downloaded from the DNA Data Bank of Japan, NCBI, the Broad Institute and HOMD database, and were used as reference genomes for short read mapping data and a count table was obtained. Then, the putative presence of *Scardovia* spp. in each sample was estimated by the following method: [*sum of reads/total number of genes ≥ 1*]. Based on this calculation, one sample was excluded from this analysis due to the low number of

reads mapped to *Scardovia*. The correlation between samples gene expression was plotted (RStudio for Mac, version 1.1.463, Plotly R package, ggplot2 R package, corrplot R package). Gene expression levels were normalized by dividing the number of reads mapping to a single gene from a bacterium by the total reads assigned to that corresponding bacterium and presented as percentages. In order to compare the gene expression of specific genes related to carbohydrate metabolism, differences in means of expression level for particular genes were tested (Wilcoxon Rank Sum Test, corresponding to the Mann-Whitney U test; RStudio, base R package; $p < 0.05$) after checking the distribution of the data (Shapiro-Wilk and Kolmogorov-Smirnov tests). The number of reads assigned to *Scardovia* spp., *Bifodobacterium* spp., *Lactobacillus* spp. and *S. mutans* in the total metatranscriptome of root caries was assessed and plotted (RStudio for Mac, version 1.1.463, Plotly R package).

Results

Pipeline a) Composition of the metabolically active microbiota and comparison with established cariogenic species and other Bifidobacteriaceae members

Figures 1 and 2 shows descriptive analyses of the number of reads in each sample in the genomes of *S. wiggsiae* and *S. inopinata* (reads of both species were summed and presented as *Scardovia* spp.), as well as species/genera conventionally recognized as cariogenic pathogens. The analyses showed a prevalence of reads assigned to *S. inopinata* and *S. wiggisae* ranging from 0 to 6% in all metatrasncriptomes of root caries. Two samples presented no gene expression of *S. inopinata* at all and only 0.04–0.07% of *S. wiggsiae*. What stands out from these charts is the very high but variable gene expression associated with *Lactobacillus* spp. This group represented 0.4% to 50.3% of all the metatranscriptome, and included the sum of reads of *Lactobacillus sp.; L. acidophilus; L. amylovorus; L. brevis; L. buchneri; L. casei; L. crispatus; L. delbrueckii; L. fermentum; L. gasseri; L. gastricus; L. helveticus; L. jensenii; L. johnsonii; L. paracasei; L.*

reuteri; L. rhamnosus; L. salivarius; amongst others. Other members of the Bifidobacteriaceae family gene expression in root caries ranged from 0.4% to 8.4% of the total metatranscriptome. This group included reads of *Bifidobacterium sp., B. dentium, B. brevis, B. longum, B. termophilum* and *Parascardovia denticolens. S. mutans* was very active in some samples (19.32% of all metatranscriptome in RC_7, for example). The most striking result to emerge from the data is that samples with *S. inopinata* gene expression representing > 5% of the total microbial metatranscriptome tended to present lower expression of *S. mutans*, but higher expression of *Lactobacillus* sp. genes (RC_A, RC_B, RC_H).

The correlation between the proportion of *Scardovia* spp. and other cariogenic species in the total metatranscriptome was tested using Pearson's correlation after checking data normality. A consistent positive correlation between *Scardovia*

spp. and *Lactobacillus* spp. was observed (r = 0.70; p = 0.03), as well as a positive correlation with other Bifidobacteriaceae members (r = 0.91; p = 0.0006). In contrast, there was a non-significant negative weak correlation of *Scardovia* spp. and *S. mutans* (r = -0.34; p = 0.35) (Figure 3).

Pipeline b) Gene expression analysis

Considering in this analysis the cut-off of the putative presence of *Scardovia* spp. assigned to each sample was the number of reads/number of genes ≥ 1, only one library had no expression of *S. inopinata* or *S. wiggsiae* (RC_E; a pool of samples of two lesions from two patients, originated from center 2). In this sample, high gene expression of *Lactobacillus spp.* and *S. mutans* was observed. One sample showed no expression of *S. wiggsiae* (RC_8; non-pooled sample, from a single patient, originated from centre 1); this was the sample with higher predominance of
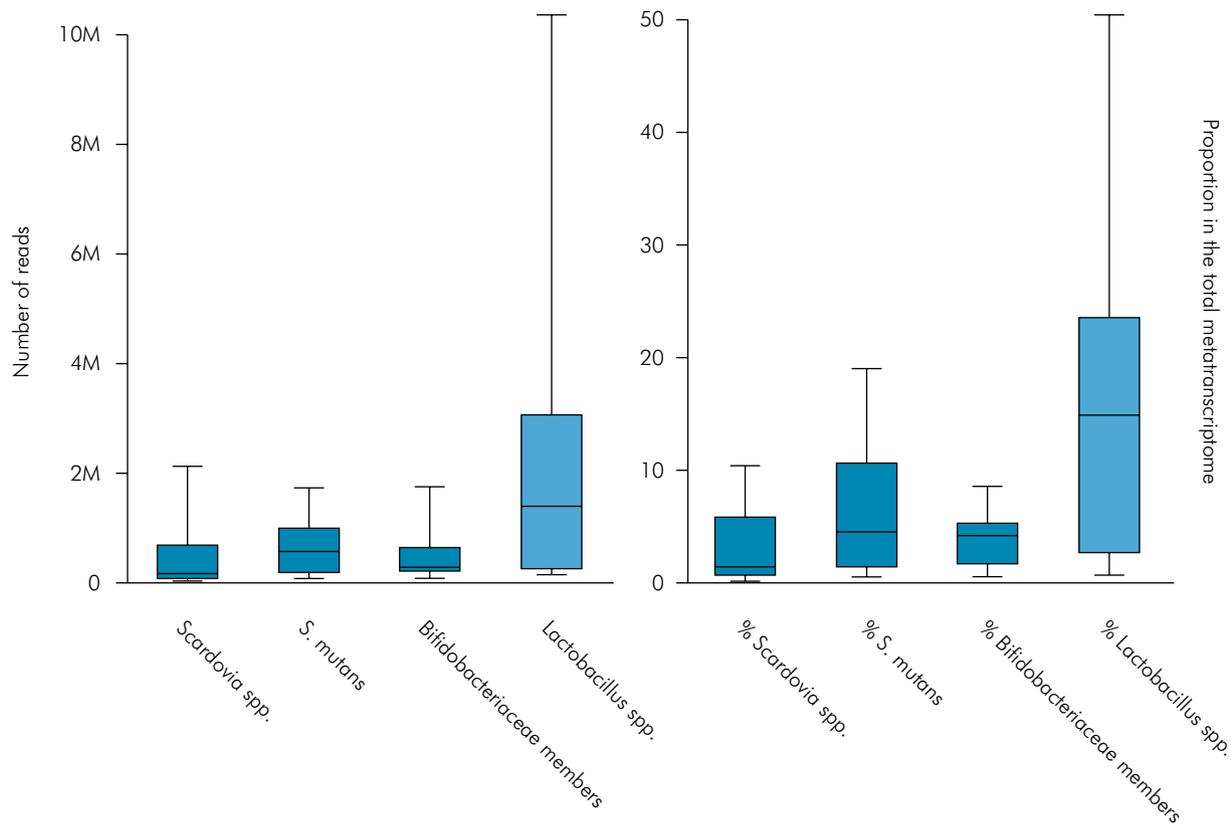

Figure 1. Number of reads and percentage in the total metatranscriptome of root caries biofilms of *Scardovia* spp., Bifidobacteriaceae members, *Lactobacillus* spp. and *Streptococcus mutans* (pipeline a).
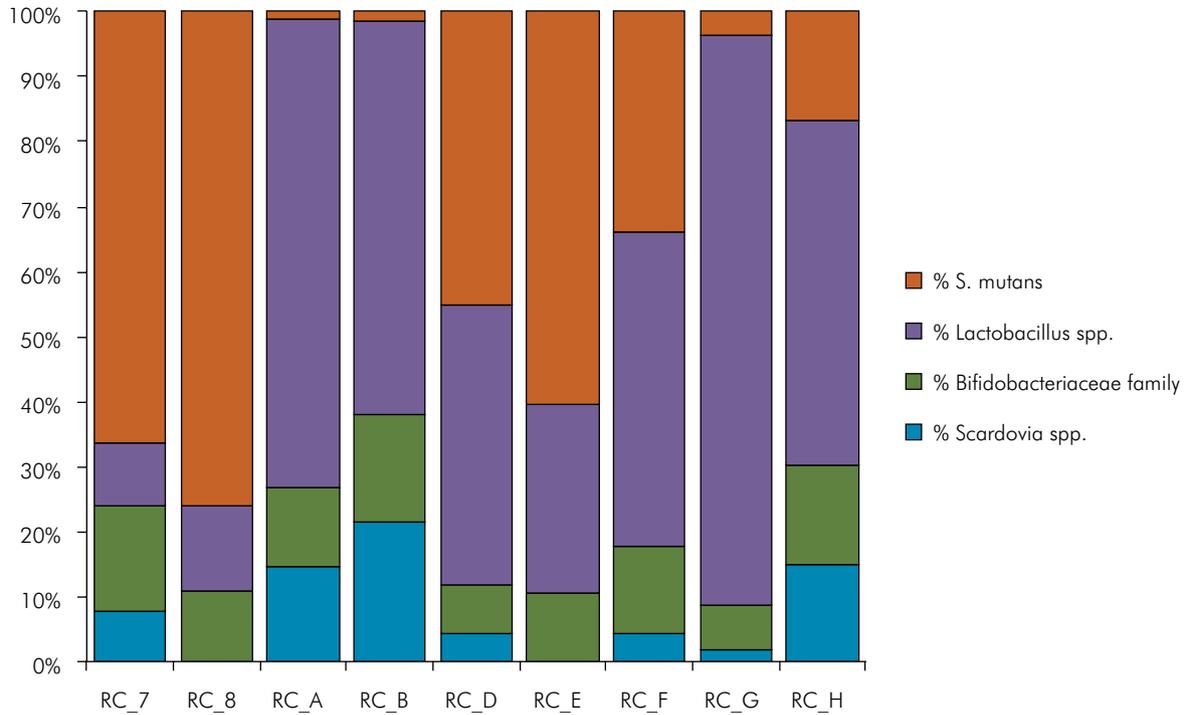
Figure 2. Proportion of reads of *Scardovia* spp., Bifidobacteriaceae members, *Lactobacillus* spp. and *Streptococcus mutans* in the metatranscriptome of root caries biofilms (pipeline a).
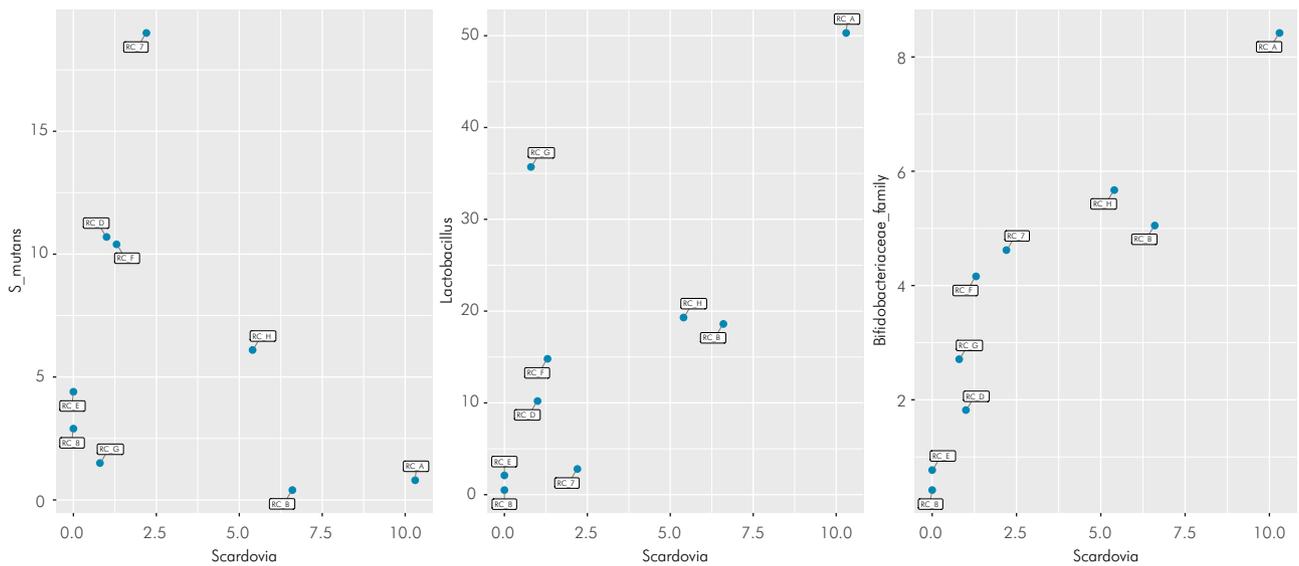

Figure 3. Correlation analysis of relative prevalence (% in the total metatranscriptome) of *Scardovia* and other cariogenic species. (pipeline a). A: *Streptococcus mutans* proportion within the total metatranscriptome; B: *Lactobacillus* species proportion within the total metatranscriptome; C: Bifidobacteriaceae family proportion within the total metatranscriptome. Each point represents each sample (RC: Root Caries).

S. mutans in the analysed species. These samples were excluded from the gene expression analysis.

Figure 4 shows the correlation matrix between samples with putative presence of *S. inopinata* (A) and *S. wiggsiae* (B) from the number of reads of all genes
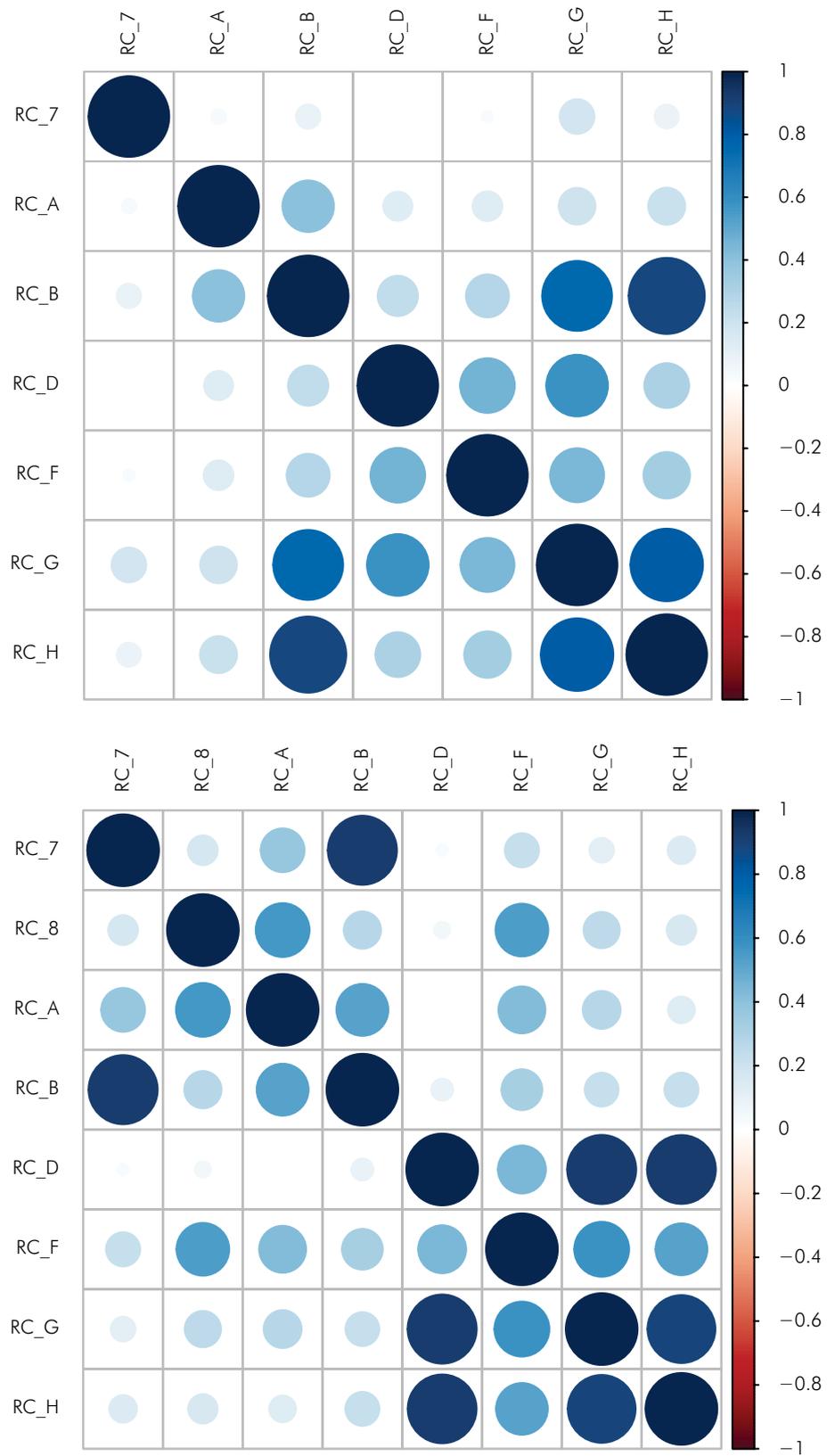
Figure 4. Degree of correlation analysis showing similarity of gene expression (number of reads) between all samples with putative presence of *Scardovia* spp. (A: *S. inopinata*; n = 8; B: *S. wiggsiae*; n = 7).

assigned to each species. From the chart, it can be seen that by far the greatest difference for both species was in the RC_D sample. This sample presented very low gene expression of *Scardovia*. Similarities between RC_7 and RC_B at *S. wiggsiae* could be observed, that was not observed for *S. wiggsiae*.

The Table shows the top 30 most expressed genes in both species. The most expressed gene represented a median of 2.2% of the transcription assigned to *S. inopinata*, that presented 170 genes with median of expression > 0.1%. While for *S. wiggsiae*, the most expressed gene represented a median of 0.68% of the transcription and 264 genes had median of expression > 0.1%, suggesting that the *S. wiggsiae* have a more complex set of functions. However, it could be observed that both species shared the majority of the highest expressed genes.

Gene expression analysis suggests the utilization of the "Bifid shunt" (oxidative pentose-phosphate (OPP) pathway) by *S. inopinata* and *S. wiggsiae* in root caries. There were differences in the ratios of expression of glucose-6-phosphate 1-dehydrogenase (median = 0.06%, 25th–75th = 0.0%–0.1%), and phosphoketolase (fructose 6-phosphate; median = 0.83%; 25th–75th = 0.2%–1.3%) (Wilcoxon test, p = 0.000). Looking at the pentose phosphate pathway enzymes (Figure 5), it appears that phosphoketolase (EC 4.1.2.2) was the most expressed enzyme in both, *S. inopitata* and *S. wiggsiae*.

Another interesting aspect of the data is the expression of genes involved in lactic acid production which were highly expressed in *S. inopinata* and *S. wiggsiae*. Formate acetyltransferase (*pfl*) was one of the most expressed genes by *Scardovia* spp. in root caries and it was expressed in all samples. The *pfl* and lactate dehydrogenase (*ldh)* genes were highly expressed by *S. inopinata* (*pfl* median = 0.5%; 25th–75th = 0.2–0.8% and *ldh* median = 0.12%; 25th–75th = 0-0.2%). *S. wiggsiae* had slightly fewer expression of both enzymes (*pfl* median = 0.26%; 25th–75th = 0.2–0.6% and *ldh* median = 0.06%; 25th–75th = 0.04–0.12%). The *pfl* was higher expressed than acetate kinase (acetate as the final end-product of the, Wilcoxon Rank Sum test; *S. inopinata* p = 0.013 and *S. wiggsiae* p = 0.029) and acetaldehyde-CoA/alcohol dehydrogenase (only present at *S. wiggsiae*) in both species (Wilcoxon Rank Sum test; *S. inopinata* p = 0.010 and *S. wiggsiae* p = 0.029).

The expression of carbohydrate-modifying enzymes was also observed. Gene expression of pullulanase by *S. inopinata* ranged from 0.2 to 0.5% (median = 0.3%; 25th–75th = 0.3%–0.4%). Pullulanase expression by *S. wiggsiae* ranged from 0.07 to 1.36% (median = 0.3; 25th–75th = 0.2–0.9). This enzyme is a glycosyl hydrolase that is responsible for the adaptation of complex carbohydrates (pullulan is produced from starch). Other glycosyl hydrolases with high gene expression included SCIP_RS00040 (median = 0.08%; 25th–75th = 0.0–0.1%), SCIP_RS05970 (median = 0.02%; 25th–75th = 0.0–0.0%), SCIP_RS04605 (median = 0.08%; 25th–75th = 0.0–0.1%), from *S. inopinata*; and HMPREF9156_RS04060 (median = 0.20; 25th–75th = 0.1–0.2%), from *S. wiggsiae.*

In the top 20 most expressed genes of *S. inopinata*, were observed the ones that code peptide ABC transporter, ABC transporter ATP-binding protein and sugar ABC transporter permease, while for *S. wiggsiae* ABC transporter ATP-binding protein (the highest median of reads in the genome), multidrug ABC transporter ATP-binding protein, ABC transporter, ABC transporter substrate-binding protein, sugar ABC transporter permease, and peptide ABC transporter ATP-binding protein featured in the list of most highly expressed genes. A gene that codes a collagen adhesion protein was also among the most expressed genes in both *S. inopinata* and *S. wiggsiae*.

Discussion

This study set out to observe the gene expression profile of *Scardovia* spp. in natural samples of root caries, and compare it with that of other well-established cariogenic microorganisms. The most obvious finding to emerge from this study is a great relative prevalence of metabolism of *S. inopinata* and *S. wiggsiae* in root caries biofilms where *Lactobacillus* spp. were also highly relatively prevalent. The research has also shown that genes with functions in some important metabolic pathways were highly expressed in root caries, such as the "bifid shunt" and starch metabolism. These findings contribute to our understanding of *Scardovia* cariogenic potential

Table. Top 30 most expressed genes of *Scardovia inopinata* and *Scardovia wiggsiae* in root caries samples.

Scardovia inopinata JCM 12537		Proportion of reads within the total reads of the genome							
Gene code	Protein	RC_7	RC_8	RC_A	RC_B	RC_D	RC_F	RC_G	RC_H
SCIP_RS06240	pyridine nucleotide-disulfide oxidoreductase	0.12	0.50	0.09	0.29	71.57	4.03	16.60	10.80
SCIP_RS04050	peptidase S8	0.24	2.97	4.82	0.56	0.43	1.63	3.38	0.79
SCIP_RS02840	phosphoketolase	0.13	1.88	0.91	0.21	0.07	2.83	1.05	0.75
SCIP_RS04235	peptide ABC transporter	0.09	1.49	2.82	0.23	0.18	0.48	2.10	0.44
SCIP_RS05945	ABC transporter	0.04	0.00	0.03	0.05	5.64	0.01	0.01	0.94
SCIP_RS00115	sugar ABC transporter ATP-binding protein	0.08	0.79	0.65	0.10	0.05	3.94	0.43	0.16
SCIP_RS03380	transaldolase	0.06	2.38	0.34	0.08	0.04	2.34	0.65	0.26
SCIP_RS03385	transketolase	0.12	2.38	0.45	0.17	0.08	1.34	1.16	0.33
SCIP_RS00890	elongation factor Tu	0.06	2.97	0.44	0.08	0.02	1.30	0.62	0.42
SCIP_RS01370	ACP S-malonyltransferase	0.60	0.10	0.92	0.66	0.18	1.88	0.61	0.96
SCIP_RS03780	formate acetyltransferase	0.16	0.99	0.68	0.19	0.08	2.23	0.63	0.37
SCIP_RS02240	ABC transporter substrate-binding protein	0.06	1.88	0.20	0.08	0.00	1.34	0.39	0.48
SCIP_RS00740	ribonucleoside-triphosphate reductase	0.16	1.98	0.42	0.18	0.02	0.38	0.55	0.73
SCIP_RS00795	collagen adhesin	0.20	0.30	0.58	0.23	0.09	1.28	1.21	0.43
SCIP_RS01120	DNA-directed RNA polymerase subunit beta	0.25	1.19	0.71	0.27	0.04	0.43	0.91	0.33
SCIP_RS04410	glyceraldehyde-3-phosphate dehydrogenase	0.07	0.40	0.37	0.10	0.05	2.16	0.49	0.22
SCIP_RS01830	type I site-specific deoxyribonuclease restriction subunit	0.18	0.00	0.00	0.00	3.07	0.05	0.02	0.45
dnaK_74	dnaK	0.18	0.20	0.21	0.14	0.00	0.26	2.56	0.14
SCIP_RS01675	ATPase AAA	0.24	0.00	0.47	0.23	0.05	1.43	1.08	0.12
fusA_113	elongation factor G fusA	0.11	1.68	0.43	0.12	0.02	0.15	0.54	0.35
SCIP_RS00770	hypothetical protein	0.16	0.69	0.27	0.15	0.02	0.61	1.03	0.17
SCIP_RS01125	DNA-directed RNA polymerase subunit beta\'	0.28	0.40	0.77	0.28	0.04	0.54	0.37	0.42
SCIP_RS06520	molecular chaperone Hsp18	0.04	0.69	0.19	0.06	0.01	0.95	0.94	0.05
SCIP_RS05935	hypothetical protein	0.09	0.00	0.11	0.10	0.00	0.01	0.01	2.59
SCIP_RS06420	hypothetical protein	0.06	0.00	0.00	0.03	0.08	0.06	0.00	2.53
SCIP_RS05960	pyridine nucleotide-disulfide oxidoreductase	0.09	0.00	0.09	0.10	1.76	0.04	0.10	0.52
SCIP_RS04220	peptide ABC transporter ATP-binding protein	0.13	1.78	0.15	0.15	0.06	0.09	0.12	0.13
SCIP_RS01555	cell division protein DivIVA	0.09	1.19	0.28	0.09	0.05	0.15	0.59	0.11
SCIP_RS02775	pyruvate kinase	0.08	0.69	0.13	0.10	0.00	1.06	0.30	0.13
SCIP_RS07225	glycogen phosphorylase	0.21	0.59	0.24	0.23	0.01	0.32	0.49	0.42
Scardovia wiggsiae F0424		Proportion of reads within the total reads of the genome							
Gene code	Protein	RC_7		RC_A	RC_B	RC_D	RC_F	RC_G	RC_H
HMPREF9156_RS06255	ABC transporter	0.38		7.48	0.54	0.20	0.30	0.06	0.04
HMPREF9156_RS06185	ABC transporter ATP-binding protein	7.10		0.05	0.17	0.04	0.15	0.32	0.15
HMPREF9156_RS06190	multidrug ABC transporter ATP-binding protein	7.19		0.11	0.14	0.02	0.15	0.22	0.15
HMPREF9156_RS06230	pullulanase	7.01		0.04	0.14	0.02	0.00	0.27	0.15
HMPREF9156_RS02600	chaperone protein ClpB	0.08		0.23	0.18	4.28	0.23	0.41	0.18
HMPREF9156_RS02030	ABC transporter substrate-binding protein	0.06		0.34	0.10	2.95	1.44	0.43	0.19
HMPREF9156_RS06215	hypothetical protein	4.80		0.08	0.06	0.02	0.08	0.11	0.09

Continue

Continuation

HMPREF9156_RS06195	endo-1,4-beta-xylanase	4.47	0.13	0.12	0.01	0.00	0.24	0.09
HMPREF9156_RS02910	ACP S-malonyltransferase	0.27	0.66	0.75	0.87	0.68	0.92	0.67
HMPREF9156_RS06220	septum formation protein Maf	4.06	0.16	0.08	0.03	0.08	0.22	0.09
dnaK_73	dnaK	0.06	0.27	0.14	3.07	0.00	0.59	0.13
HMPREF9156_RS01510	phosphoketolase	0.07	0.28	0.24	1.36	1.59	0.45	0.24
HMPREF9156_RS04005	sugar ABC transporter ATP-binding protein	0.01	0.17	0.09	2.66	0.84	0.35	0.11
HMPREF9156_RS00765	formate acetyltransferase	0.12	0.25	0.20	1.94	0.99	0.46	0.27
HMPREF9156_RS06235	hypothetical protein	3.79	0.03	0.11	0.01	0.00	0.18	0.11
HMPREF9156_RS03785	peptidase S8	0.21	0.68	0.40	1.35	0.46	0.60	0.44
HMPREF9156_RS06180	hypothetical protein	3.53	0.08	0.09	0.02	0.00	0.19	0.10
HMPREF9156_RS02025	sugar ABC transporter permease	0.12	0.28	0.11	1.10	1.82	0.27	0.16
HMPREF9156_RS06210	homoserine dehydrogenase	3.18	0.05	0.07	0.03	0.15	0.14	0.07
HMPREF9156_RS03140	pullulanase	0.18	0.53	0.40	0.83	0.91	0.40	0.39
HMPREF9156_RS06205	2-hydroxyhepta-2,4-diene-1,7-dioate isomerase	3.17	0.07	0.08	0.02	0.00	0.05	0.06
HMPREF9156_RS06270	hypothetical protein	0.07	0.25	0.10	0.84	1.59	0.25	0.18
HMPREF9156_RS04820	hypothetical protein	0.13	0.41	0.35	0.74	0.53	0.61	0.32
HMPREF9156_RS03425	elongation factor Tu	0.06	0.29	0.08	0.86	1.21	0.34	0.15
HMPREF9156_RS00310	glyceraldehyde-3-phosphate dehydrogenase	0.09	0.07	0.10	1.28	0.99	0.31	0.10
HMPREF9156_RS05500	ATP-dependent Clp protease ATP-binding subunit ClpC	0.08	0.25	0.16	1.77	0.15	0.31	0.19
HMPREF9156_RS04550	glycogen phosphorylase	0.15	0.16	0.29	0.44	1.29	0.26	0.26
rpsN_43	rpsN	0.34	0.05	0.01	0.05	2.35	0.02	0.01
HMPREF9156_RS06200	YggS family pyridoxal phosphate enzyme	2.54	0.03	0.06	0.01	0.00	0.14	0.05
HMPREF9156_RS03180	DNA-directed RNA polymerase subunit beta\'	0.06	0.27	0.28	0.84	0.53	0.49	0.26

and provide a basis for new investigations of their role in dysbiosis.

Many species have been found in root caries lesions, but studies are still focusing on well-established cariogenic microorganisms, such as *S. mutans* and *Lactobacillus* spp. However, previous studies reported low or highly variable percentages of these predicted cariogenic species in root caries [16,21,22]. Although culture and DNA-based studies showed low abundance of *Lactobacillus* sp. and *S. mutans*, the RNA-seq showed great gene expression here. Such approaches have also failed to address *Scardovia* spp. prevalence. In this study, the proportion of each, *S. inopinata* and *S. wiggisae*, reached approximately 6% of the total metatranscriptome of root caries. Scardovia has been detected in caries lesions,[12,13,14] in association with root caries progression.[9,10,16] It was defined as one of the organisms that could

be a 'keystone' for coronal caries.[15] It is interesting to note that only one sample had no expression at all of both species, and all other samples in the present study had activity of at least one species of Scardovia. In this sample, a great gene expression of *Lactobacillus* spp. and *S. mutans* was observed. We also sequenced biofilm samples from sound root surfaces; however, using the cut-off, only one out of 10 samples presented Scardovia (data not shown), suggesting that its prevalence is related to the root caries environment.

The correlation of the proportion of *Scardovia* within the metatranscriptome was evaluated and a significant positive correlation with *Lactobacillus* and Bifidobacteriaceae members was observed, while a non-significant negative correlation with *S. mutans* was found. Nyvad et al.[23] characterized the root caries microbiota *in vivo*, suggesting that a dominance of

a single or few microorganisms may reflect a very high cariogenicity. This is in line with the results of our study, which showed a predominance of one or few species, and a pattern of predominance of *Lactobacillus* spp. with others, but *S. mutans* appeared to be active as the main cariogenic species in the community. Eriksson et al.[24] suggested that in subjects with caries experience, high levels of *S. mutans* were associated with the presence of a few saccharolytic species, including *S. wiggsiae*. Similarly, a culture-based study showed that the presence of both *S. wiggsiae* and *S. mutans* was associated with severe early childhood caries.[13] Another previous *in vitro* study showed that *Scardovia* acid production was higher when in association with *S. mutans*.[11] However, our results showed that clinically this association is not preferable from both species regarding their metabolism in root caries.

Bifidobacteria possess a high number of enzymes involved in sugar metabolism.[25] Phosphoketolase (Figure 5) is the key enzyme for the "bifid shunt" and both species of *Scardovia* possess it. This pathway allows the production of more energy from carbohydrates compared with that produced by the Embden-Meyerhof-Parnas pathway.[26] Phosphoketolase is also a taxonomic marker for

the family of Bifidobacteriaceae. Our results showed that this enzyme was the highest expressed in the OPP pathway enzymes in both *S. inopinata* and *S. wiggsiae* (Figure 5).

In general, high lactate production correlated with low amounts of acetate, ethanol and formate production and vice versa,[27] and it is caused by the rapid consumption of an energy source.[18,26] We observed high gene expression of lactate dehydrogenase (*ldh*) and pyruvate formate lyase (*pfl*) in *Scardovia*. Furthermore, the *pfl* was significantly higher expressed than acetate kinase (in both species) and acetaldehyde-CoA/ alcohol dehydrogenase (only at *S. wiggsiae*), which could result in a higher concentration of lactate than acetate and alcohol production by these species, and this may be related to root caries progress.

The expression of carbohydrate-modifying enzymes was also observed. As all samples presented an expression of at least 0.2% of the whole transcriptome of the species, it suggests that this is an essential gene to survive *in vivo* in root caries. Pullulan is a polysaccharide polymer consisting of maltotriose units, and it is produced from starch and, thus, *Scardovia* may be metabolizing starch that is cariogenic to root caries.[28]
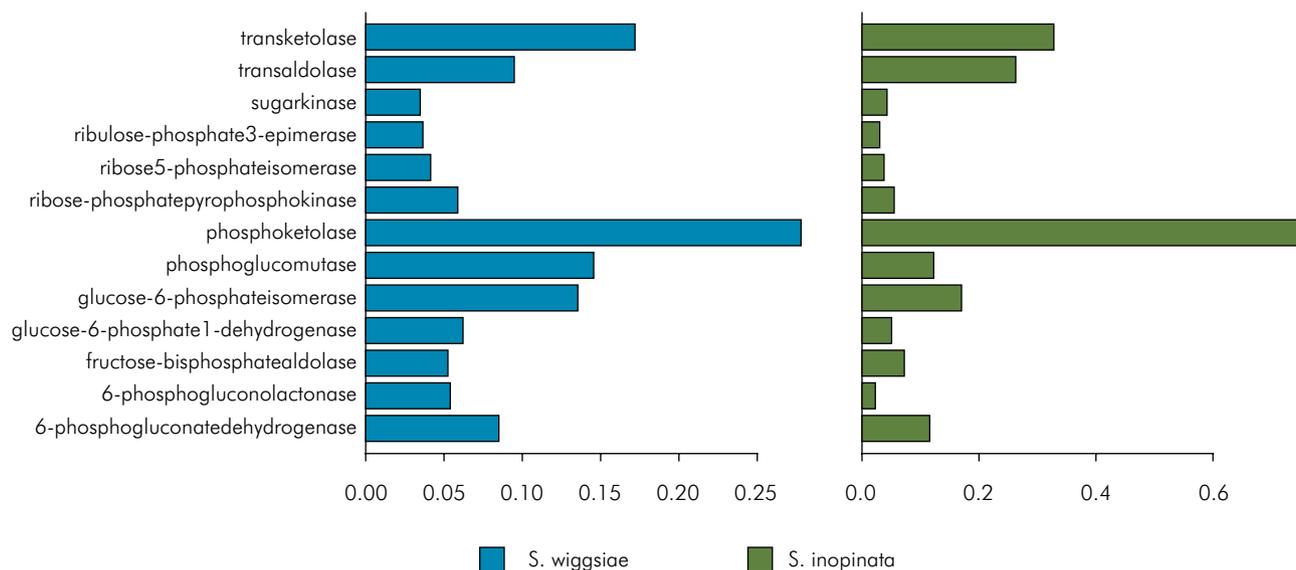

Figure 5. Proportion of reads assigned to genes that code Oxidative Pentose Phosphate Pathway enzymes in the transcriptome of *Scardovia* species. Median of gene expression at n = 8 samples with putative presence of *Scardovia*.

The literature has shown that the gene content of a bifidobacterial genome seems to reflect its adaptation, as indicated by the presence of genes that encode a variety of carbohydrate-modifying enzymes. As expected, many genes coding ABC transporters had high levels of expression in both species. ABC systems are responsible for the transport of nutrients such as mannose-containing oligosaccharides, while glucose is internalized using a glucose-specific PEP:PTS.[18,26] It could suggest that in root caries, *Scardovia* spp. use low glucose but high complexity carbohydrates because it expresses more ABC transporters than PEP:PTS systems. In contrast to *S. mutans*, which possess 14 PEP:PTS systems, only a minority of the sugars utilized by bifidobacteria are internalized via PEP:PTS.[18,26]

Strengths of this study include the type of methodology used (RNA-seq). As the target molecule of this study was mRNA, the result represents the proportion of general metabolic activity. The metatranscriptome allows the investigation of a large number of genes; however, specific aspects of how some species collaborate to promote root caries progression, as showed here, would be unmanageable by showing the results of the metatranscriptome as a whole. This account must be approached with some caution because it was based on short read mapping and this could add some bias in analysing reads at the species-level. This led us to use two different analysis pipelines to try overcome this issue. The contigs with ~500bp were assembled and mapped to species.

A number of conclusions can be drawn from the present descriptive study. There is a positive correlation between the total metabolism of *Lactobacillus* spp., other Bifidobacteriaceae and *Scardovia* sp. in root caries, which could suggest a symbiotic relationship between these species. The Bifid shunt and starch metabolism are expressed in *S. inopinata* and *S. wiggsiae* in root caries. The understanding of *Scardovia* sp. as a protagonist in root caries might provide meaningful information for the development of future strategies of diagnosis and treatment.

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