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Acid tolerance of *Lactobacillus* spp. on root carious lesions: a complex and multifaceted response

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Summary declaration of interest statement

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Abstract

Lactobacillus spp. are acidogenic and aciduric bacteria and are among the main cariogenic microorganisms associated with the carious process. **Objective:** This study aimed to identify genes involved in the acid-tolerance of *Lactobacillus* spp. and potential functions attributed to these genes within the metatranscriptome of sound root surfaces and carious root surfaces. **Design:** Genomic libraries were built from mRNA isolated from the biofilm samples (10 from sound root and 9 from carious root using Illumina HiSeq 2500). Reads generated by RNA-seq were mapped against 162 oral microbial genomes and genes potentially related to acid tolerance were manually extracted from the *Lactobacillus* spp. genomes using *L. paracasei* ATCC 344 as reference genome. The R package DESeq2 was used to calculate the level of differential gene expression between those two clinical conditions. **Results:** Fifteen *Lactobacillus* spp. genomes were identified and a total of 653 acid tolerance genes were overexpressed in carious root surfaces. Multiple functions, as translation, ribosomal structure and biogenesis, transport of nucleotides and amino acids, are involved in *Lactobacillus* spp. acid tolerance. Species-specific functions also seem to be related to the fitness of *Lactobacillus* spp. in acidified environments such as that of the cariogenic biofilm associated with carious root lesions. **Conclusions:** The response of *Lactobacillus* spp. to an acidic environment is complex and multifaceted. This finding suggests several possible avenues for further research into the adaptive mechanisms of these bacteria.

Keywords: *Lactobacillus*, transcriptome, Illumina, dental caries, biofilm, acid tolerance

Introduction

Root caries, which is defined as a carious lesion that affects the root surface of the tooth after being exposed to the oral environment due to gingival recession (Ritter et al., 2010; Tan et al., 2017) is increasingly prevalent worldwide (Hariyani et al., 2018) as a consequence of the increase in life expectancy as well as of the reduction in edentulism at all ages (Pentapati et al., 2019; Tan et al., 2017). A dysbiotic dental biofilm, often exposed to fermentable carbohydrates, can contribute to the development of root carious lesions through a two-stage process. The initial stage involves demineralization, while the subsequent stage encompasses the degradation of the collagen matrix (Takahashi & Nyvad, 2016).

Dental biofilm is a highly diverse polymicrobial community (Bowen et al., 2018) and some of its members have the ability to produce acids from the metabolism of carbohydrates which decrease the pH at the tooth/biofilm interface predisposing tooth surface to mineral loss (Takahashi & Nyvad, 2008). Additionally, low pH induces changes in the biofilm's microbial composition leading to a dysbiotic state characterized by the selection of and by the greater abundance of highly acidogenic and aciduric microorganisms that in turn results in more acidic environments perpetuating the mineral imbalance at the tooth surface (Takahashi & Nyvad, 2016).

Lactobacillus spp. is within the acidogenic and aciduric genera found in dental biofilms (Caufield et al., 2015; Wen et al., 2022), comprising *L. fermentum*, *L. rhamnosus*, *L. gasseri*, *L. casei/paracasei*, *L. salivarius, L. plantarum* species most frequently isolated from saliva, biofilm or from carious dentine of individuals presenting dental caries (Caufield et al., 2015; Piwat et al., 2010; Wen et al., 2022). *L. casei*, *L. crispatus, L. paracasei,* and *L. rhamnosus* were also among the most frequent species identified in individuals presenting root carious lesions (Chen et al., 2015; Preza et al., 2009). In this context, salivary levels of *Lactobacillus* spp. seem to be higher in adults presenting active coronal carious lesions (Sounah & Madfa, 2020) and a positive correlation between caries experience and salivary levels of *Lactobacillus* spp. in mixed and in permanent dentitions were also reported (Chokshi et al., 2016). In relation to root caries, a strong correlation between salivary levels of *Lactobacillus* and the number of root carious lesions has been

reported (Beighton et al., 1991). Moreover, *Lactobacillus* spp. were the predominant aciduric bacteria isolated from dentinal samples collected from carious root surfaces (Brailsford et al., 2001). It seems that *L. casei, L. paracasei and L. rhamnosus* are notably associated with carious root surfaces (Preza et al., 2009). Since they are not able to efficiently adhere to smooth surfaces, cavitated and/or retentive dental sites are the preferred ones for colonization of *Lactobacillus* spp. Many species of this genus are able to actively adhere to dental type I collagen that is exposed to the oral cavity both under sound root surface exposure through gingival recession and during the progression of dentinal carious lesion (Caufield et al., 2015; Wen et al., 2022). Thus, there is an understanding that these microorganisms are more related to the progression of an existing carious lesion than involved in the development of initial and non-cavitated ones (Caufield et al., 2015; Wen et al., 2022).

Lactobacillus spp. aciduricity is exerted by several constitutive and adaptive mechanisms, such as the ATPase-dependent proton translocation pump (also referred as H+ ATPase pump), the production of alkaline/neutralizing molecules via urease activity, aspartate metabolism, arginine and agmatine deiminase systems, the action of two-component system the production of acid shock proteins as well as the decarboxylation of glutamate. The literature indicates the expression of such mechanisms differs among different lactobacilli species (Broadbent et al., 2010; Guan & Liu, 2020; Papadimitriou et al., 2016; Wu et al., 2012, 2013).

Although the individual importance of such mechanisms for *Lactobacilli* acid tolerance has been clearly shown under well-controlled *in vitro* conditions, it is still unclear how those mechanisms are expressed among distinct lactobacilli species under clinical conditions compatible with dental caries development. In this context, comparative metatranscriptome analysis of dental biofilm collected from sound root surfaces and from carious root surfaces allow the identification of species-specific differentially expressed genes and their associated functional patterns which could be indicative of functional signatures associated with sound or with carious surfaces. Therefore, the aim of this study was to identify genes and their putative functions related to *Lactobacillus* spp. aciduricity within the metatranscriptome of biofilms from sound- and from carious root surfaces.

Materials and methods

Patient selection, sample collection and sample preparation for this RNA-Seq study were performed and detailed described elsewhere (Dame-Teixeira et al., 2016). In short, adult individuals, men and women, mean age of 60.1 ± 11.6 years (40 – 90), not wearing dentures, not undergoing/not underwent antibiotics, not undergoing/not underwent radiotherapy to the head and neck, who had at least one exposed sound root surface (n = 10) or who had at least one primary active cavitated root lesion that needed restorative treatment (n = 30) were recruited. All carious lesions showed clinical characteristics of lesion activity (wet/soft dentine consistency with yellowish color) (Nyvad & Fejerskov, 1982). Dental biofilms (from sound surfaces) and biofilm/dentine (from the carious surfaces) were collected from all available exposed root surfaces with sterile Gracey 3-4 curette. All samples from carious surfaces were collected from the participants during the treatment of caries disease. All participants were asked to refrain from tooth brushing for at least 12 hours prior to the sampling, to allow for dental biofilm accumulation, and were also asked to refrain from eating and drinking for at least 1 hour prior to the sampling. Participants of 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.

Biofilm and biofilm/dentine samples were immediately placed at RNAprotect reagent (QIAGEN, Inc., Venlo, Netherlands). Total RNA was extracted from all samples using an UltraClean Microbial RNA isolation kit (Mo-BIO Laboratories, Inc., SanDiego, CA), in a column for DNAse digestion (QIAGEN, Inc., Venlo, Netherlands). RNA was quantified using the Quant-iTTM RiboGreen® RNA Assay Kit (Invitrogen, Inc., Waltham, Massachusetts, USA). The yield of RNA from carious dentine samples is lower than from samples containing only biofilm. Since a minimum of 30 ng/RNA is necessary for proper sequencing of the samples, those with total RNA concentration <30 ng / RNA were pooled. This process resulted in 10 samples from the sound root surfaces group and 9 samples from the carious root surfaces. The Ribo-Zero ™ Meta-Bacteria kit (Epicenter, Illumina) was

used to enrich mRNA and preparation protocols from the Illumina®TruSeq ™ library (Illumina, San Diego, CA) were used to prepare and sequence the library with Illumina HiSeq2500. The FASTQ files were obtained for each sample and mapped against 162 oral microbial genomes using the Qiagen CLC Genomics Workbench (Dame-Teixeira et al., 2016). Complete data is available at the National Center for Biotechnological Information (NCBI), under accession numbers SRS779973 and SRS796739. This study was conducted ethically in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki). This study protocol was reviewed and approved by the Ethics Committee of the Federal University of Rio Grande do Sul (protocol 8.427.168) and by the National Research Ethics Service Committee Yorkshire & the Humber – Leeds West (protocol 8 2012002DD). All participants consented to donate samples after reading and signing an informed consent.

Genome and analysis of Lactobacillus spp.

The genes of all *Lactobacillus* spp. were then manually extracted from the genomes found in the in samples. The putative presence of *Lactobacillus* genomes in the samples was determined by the ratio between the total number of mRNA reads of each sample and the total number of identified genes in the genome of interest. Genomes were considered present whether this ratio was above 1.0 (Dame-Teixeira et al., 2016).

Acid tolerance genes were identified on the assessed genomes using *L. casei* ATCC 344 as reference. This microorganism was chosen because a comprehensive analysis of its transcriptional response to acid stress is available for comparison. In the study of (Broadbent et al., 2010), one hundred-eighty *L. casei* genes were potentially associated with response to acidic environments.

Data analysis

All genes belonged to the 162 genomes found in the assessed samples were submitted to the DESeq2 R package to obtain the differentially expressed genes (DEGs) between sound root surfaces and carious root surfaces (Love et al., 2014). Subsequently,

genes potentially related to acid tolerance were manually extracted from all *Lactobacillus* spp. genomes. The cutoff point for being considered as DEGs was a change in transcription levels of at least 1 Log2fold change using the Benjamin and Hochberg method to multiple testing correction (Love et al., 2014) and the adjusted p-value (padj) to be less than 0.001. This high cut-off point was chosen to avoid false-positive results. Negative values indicated genes overexpressed in sound root surfaces while positive values meant overexpressed gene in the carious root surfaces. The biological processes related to the DEGs were obtained through the KEGG platform (Kyoto encyclopedia of genes and genomes) and gene information was obtained from the UniProt platform.

Results

In total, fifteen genomes of *Lactobacillus* spp. belonging to the following species were identified in the samples: *L. acidophillus, L. brevis, L. buchneri, L. casei, L. crispatus, L. curvatus, L. delbrueckii, L. fermentum, L. gasseri, L. jensenii, L. johnsonii, L. paracasei, L. plantarum, L. rhamnosus* and *L. salivarius*. Putative presence of *Lactobacillus* spp. (i.e, *L. fermentum, L. gasseri* and *L. paracasei*) was found in sound root surfaces of only one participant (SRS_12; mRNA read count ranged from 1.67 to 8.26) **(Table S1**). On the other hand, *Lactobacillus* spp. were putatively present in all carious root surfaces except in those from one participant (RC_8). mRNA read count on carious root surfaces ranged from 1.22 to 3,582.72 and the mean (±sd) read count per sample was 88.94±362.32. **(Table S2)**. In general, a mean of 2,137.46±457.85 genes were found per genome.

From a total of 32,062 genes assigned to the identified *Lactobacillus* genomes, 7,868 were differentially expressed. A total of 1,737 genes (a mean of 115.8±23.26 genes per genome) were identified as potentially related to acid tolerance. All 653 acid tolerance DEGs showed Log2fold change >1 indicating an overexpression in carious root surfaces. The percentage of acid tolerance DEGs in relation to all DEGs ranged from 4.76 to 57.14% being this estimative highly variable among the genomes **(Table 1)**. *L. rhamnosus* and *L. salivarius* presented the highest number of acid-tolerance DEGs whereas only one acid tolerance DEG was found on *L. casei* genome (**Table 1**). All acid tolerance DEGs per each genome, along with their Log2fold change values, p value, as well as the respective biological process and the protein name encoded by those genes are shown in **Table S3**.

Overall, translation and biogenesis represented most of the processes attributed to the acid tolerance DEGs, followed by nucleotide transport and protein repair, whereas carbohydrate transport and unknown functions corresponded to less than 1% of the biological processes attributed to the acid tolerance DEGs **(Figure 1).** The contribution of the biological processes to the acid tolerance varied among the *Lactobacillus* ssp. genomes **(Figure 2)**. Translation and biogenesis, nucleotide transport and energy production were the most abundant processes identified in most of the genomes. Fifty percent of the acid tolerance DEGs in *L. plantarum* corresponded to nucleotide transport and metabolism process. Nucleotide transport and metabolism, DNA elements and signal transduction process contributed equally for *L. curvatus* acid tolerance, whereas translation and biogenesis, and nucleotide transport and metabolism seemed to be the most elicited ones in *L. paracasei*. Nucleotide transport and metabolism processes was the second most abundant one attributed to the DEGs in *L. crispatus, L. gasseri, L. rhamnosus* and *L. salivarius* (corresponding from 9.89 to 18.1% of DEGs), whereas energy production and conversion functions were the second most present functions in *L. acidophillus* and *L. brevis* (corresponding from 18.1 to 21.2% of DEGs). The second most abundant functions in *L. buchneri* and in *L. johnsonii* was proteins repair (from 14.6 to 25% of DEGs), but the functions of amino acid transport and metabolism were the second most abundant ones in *L. delbrueckii* and in *L. jensenii* (in both genomes corresponding to 25% of DEGs). Functions of DNA elements and translation and ribosomal structure and biogenesis were the second most abundant functions in *L. fermentum* and in *L. plantarum* the, respectively (**Table S4**). **Figure 3** summarizes the main biological processes shared by *Lactobacillus* spp. genomes.

Acid tolerance DEGs that presented the highest Log2fold change values in each genome are described in **Table S4**. The highest value was found in *L. crispatus* (12.54; gene *LCRIS_RS01510*) while the lowest one was found in *L. casei* (6.73; gene *LBCZ_RS05780*). Among all these genes, 5 were associated with translation function, ribosomal structure and biogenesis (*L. casei LBCZ_RS05780*, *L. crispatus LCRIS_RS01510*, *L. johnsonii LJ_RS01875*, *L. plantarum JDM1_RS04365* and *L. rhamnosus LGG_RS11910*), 3 genes were associated with DNA elements (*L. curvatus OA78_RS05010*, *L. delbrueckii LDB_RS07740* and *L. fermentum LAF_RS01020*), 2 genes

were associated with repair proteins (*L. brevis LVIS_RS14560* and *L. salivarius HMPREF0545_RS03630*), 1 gene was related to energy production and conversion (*L. acidophilus LBA0774*), to nucleotide transport and metabolism (*L. buchneri LBUCD034_RS02605*), 1 gene was related to cell envelope biogenesis (*L. gasseri* LGAS_RS05580), 1 gene was related to amino acid transport and metabolism (*L. jensenii* HMPREF0526_RS06110) and 1 gene was related to protein acyltransferase function (*L. paracasei* LSEI_1868)). Three out the fifteen above mentioned genes had their metabolic pathways available on KEGG and on Uniprot plataforms: *atpF* (*LBA0774*) is related to the synthesis of the B subunit of the ATP synthase protein that is involved in the function of the F1F0 ATP synthase pump (energy production); *rplC* (*LCRIS_RS01510*) encodes an L3 protein of the ribosomal structure and *rfbB* (*LGAS_RS05580)* is related to the transporter route of ABC membrane.

Discussion

Culture-independent-based data showed that caries-free individuals present higher levels/abundance of *Bacteroidetes*[G-2] sp., *Capnocytophaga* spp., *Delftia acidovorans, Fusobacterium nucleatum* subsp. polymorphum, *Kingella oralis, Lachnospiraceae*[G-3] sp., *Leptotrichia* spp., *Prevotella intermedia, Selenomonas noxia*, *Streptococcus* spp., *Streptococcus cristatus,* and *Veillonella* spp. while individuals with root caries present higher levels/abundance of not only *Lactobacillus* spp., but also of *Atopobium* spp. *Bifidobacterium* spp., *Olsenella profusa*, *Prevotella multisaccharivorax, Propionibacterium* spp. *Pseudoramibacter alactolyticus* and *Streptococcus* spp., including *Streptococcus mutans* and *Streptococcus sobrinus* (Chen et al., 2015, 2023; Hashimoto et al., 2011; Preza et al., 2008). Regarding the functional profile, the transcriptome of some microorganisms within the metatranscriptome of root caries showed an enrichment of patterns related to sugar metabolism, cell-wall biosynthesis and to acid tolerance stress (for *S. mutans*), overexpression of genes related to mobile elements, ribosome, transcriptional regulators, polysaccharide biosynthesis, among others (for *Actinomyces* spp.) and to metabolic activity, sugar transport, stress tolerance, invasion and pH regulation (Dame-Teixeira et al., 2016; Ev et al., 2020; Santos et al., 2022). As previously reported, *Lactobacillus* spp. comprise from 0.4% to 50.3% of the metatranscriptome of carious root

surfaces, presenting a high and variable gene expression. Moreover, *Lactobacillus* spp. proportion was positively correlated with *Scardovia* spp. and with Bifidobacteriaceae members (Damé-Teixeira et al., 2020). Our data shows that biofilms/dentine collected from carious root lesions presents high diversity of *Lactobacillus* spp. This study identified fifteen genomes in carious root surfaces which agrees with previous studies showing a high abundance of *Lactobacillus* spp. on root carious lesions samples (Brailsford et al., 2001; Li et al., 2015; Preza et al., 2009; Dame-Teixeira, et al., 2020; Wattanarat et al., 2020; Wen et al., 2022). Altogether, these data indicate that retentive site of carious root surfaces, along with the *Lactobacillus* spp. high acid tolerance, facilitate and promote lactobacilli adhesion to and colonization of carious root surfaces (Li et al., 2015; Wen et al., 2022), which might explain the putative absence of those microorganisms on sound root surfaces. Moreover, the higher levels of *Lactobacillus* spp. on individuals with root caries may also be the result of an ecological shift due to dental caries development.

Acid-tolerance is an inherent protective trait both for microorganisms that are thrilled by acidic environments as well as for acid-lactic producing ones. In this sense, the acid-tolerance of *Pichia kudriavzevi*, a microorganism isolated from Chinese soy fermented grains, is associated with the overexpression of genes related to ABC-transport proteins (Du et al., 2022). An increased transcription of genes encoding proton-pumping enzymes, as well as, the cell-wall fatty acid content rearrangement were observed in *Saccharomyces cerevisiae*, a yeast commonly used for fermentation of food and beverages (Deng et al., 2020; Peetermans et al., 2021). Cell-wall fatty acid remodeling is also responsible for allowing the growth of probiotic *Bifidobacterium longum* and the vinegar contaminant *Acetilactobacillus jinshanensis* subsp. *aerogenes* under acidic conditions (Li et al., 2023; Liu et al., 2016). Acid tolerance of two pathogens associated with food-poisoning events (*Bacilus cereus* and *Salmonella typhimurium*), of *S. cerevisiae* and of *A. jinshanensis* subsp. *aerogenes,* is also dependent on the upregulation of genes responsible for biosynthesis, metabolism and transport of amino acids (Álvarez-Ordóñez et al., 2010; Li et al., 2023; Peetermans et al., 2021; Senouci-Rezkallah et al., 2011). Moreover, the acid tolerance of *Listeria monocytogenes*, a human pathogen found in food-processing environments, is also granted by the glutamate decarboxylase, arginine deiminase, as well as H^+-ATPase pump activities (Ryan et al., 2008; Smith et al., 2013).

The ability to thrive on acidic environments is also an evolutionary trait of oral microorganism enabling them to colonize tooth surfaces (Badet & Thebaud, 2008; Broadbent et al., 2010). Acid stress induces an increase not only in the activity of H^+-ATPase pump and in the arginine deiminase system, but also on the levels of stress-related proteins, such as heat shock ones in non-mutans streptococci (*Streptococcus sanguinis, Streptococcus gordonii, Streptococcus oralis* and *Streptococcus mitis* (Takahashi & Yamada, 1999). Urea degradation seems also to be an important mechanism used by *Streptococcus salivarius* to cope with acidic environments (Quivey et al., 2000). Acid tolerance response has been also widely studied in *S. mutans*. It has been shown that several mechanisms are responsible for its high aciduricity, as extrusion of protons by H^+-ATPase pump (Baker et al., 2015, 2017; Gong et al., 2009), shifts in cell wall fatty-acid composition (Baker et al., 2015; Fozo et al., 2004; Gong et al., 2009), agmatine degradation (Baker et al., 2017), degradation of low pH altered proteins, among others (Lemos et al., 2005). Arginine biosynthesis and urea catabolism pathways were also upregulated in *Actinomyces* spp. within the metatranscriptome of root caries (Dame-Teixeira et al., 2016). By comparing with all DEGs, our metatranscriptome data show that only 8.29% of actively transcribed genes are associated with lactobacilli acid tolerance functions. All those acid tolerance DEGs were upregulated on carious root surfaces and they contribute to acid tolerance through distinct biological processes.

Translation and biogenesis, nucleotide transport and energy production were the main functions associated with most of the *Lactobacillus* spp. overexpressed genes in the carious root surfaces. Together, these functions corresponded to 69.37% of all acid tolerance related DEGs **(Figure 1)**. Fifty-three DEGs were related to energy production and to ATP synthase components, such as the *L. acidophilus atpF* gene and other genes of *L. brevis, L. buchneri, L. crispatus, L. fermentum, L. gasseri, L. jensenii, L. johnsonii* and of *L. paracasei* **(Tables S3 and S4)**. The hydrogenionic gradient between intracellular and extracellular compartments enables ATP molecules synthesis at the time these ions diffuse into the intracellular compartment. Part of those ATP provides energy for proton extrusion to the extracellular environment via H+ ATPase pump in order to control the intracellular pH (Broadbent et al., 2010). This is an important mechanism of acid tolerance response also observed in *S. mutans* (Quivey et al., 2016). Moreover, it has also been reported that

this acid tolerance mechanism may be favored by malolactic fermentation, in which malate decarboxylation allows the generation of ATP also through H+ ATPase pump (Broadbent et al., 2010). This is supposed to be one of the acid tolerance mechanisms of *L. fermentum* which showed an overexpression of gene LAF_RS05590 that codes for malate dehydrogenase **(Table S4)**. Besides, ninety-two DEGs were associated with transport and metabolism of nucleotides, such as *purB* gene of *L. buchneri* related to purine biosynthesis, and other genes related to transcription elongation factor, transferases, reductases, hydrolases, decaboxylases, lyases and kinases of *L. acidophilus, L. crispatus, L. curvatus, L. delbrueckii, L. fermentum, L. gasseri, L. paracasei, L. plantarum, L. rhamnosus* and *L. salivarius* (**Tables S3 and S4**). We hypothesized that the synthesis of adenine, a type of purine, is also related to ATP generation that provides energy for H+ ATPase pump activity, reinforcing the importance of these biological processes for eliciting microbial acid tolerance. In fact, nucleotide transport and metabolism were one of the most abundant functions found in seven of the analyzed genomes (*L. curvatus, L. plantarum, L. paracasei, L. crispatus, L. gasseri, L. rhamnosus* and *L. salivarius*) (**Figure 3**) (Baker et al., 2017) .Collectively, these results mean that the main basic cellular processes, as macromolecule synthesis, especially proteins and nucleic acids, and energy production are needed for microbial fitness to acid environment. These functions are shared by almost all evaluated genomes (**Figure 3**).

An overexpression of genes related to amino acid transport and metabolism has been previously described when *L. casei* is cultivated *in vitro* at pH 4.5 over a short period of time (Broadbent et al., 2010). Synthesis of lysine promotes strong tolerance for *L. delbrueckii* subsp *bulgaricus* under acidic conditions (Li et al., 2020). These findings agree with our data which showed 59 DEGs on carious root surfaces that are associated with amino acid transport and metabolism. It has been reported that glutamate decarboxylase system plays an important role on *L. brevis* acid tolerance (Lyu et al., 2018). On possible explanation for this increased gene expression is that amino acid uptake also contributes to intracellular pH homeostasis, especially providing glutamate, arginine and lysine for decarboxylation processes (Álvarez-Ordóñez et al., 2010; Peetermans et al., 2021; Senouci-Rezkallah et al., 2011). ABC transporters, ABC permeases, amino acid- and oligopeptides-transporters, and decarboxylase, transaminase, synthetase and aminopeptidase activities are

some encoded functions attributed to the DEGs of *L. acidophilus, L. buchneri, L. crispatus, L. delbruecki, L. fermentum, L. gasseri, L. jensenii, L. paracasei, L. rhamnosus* **(Table S3)**. Among all the assessed genomes, *L. jensenii* showed the gene *HMPREF0526_RS06110* as the one with the highest Log2fold change value (Table S4). Besides, this increase on amino acid transport may be directly related to the general increase on translation and biogenesis processes described above.

Fatty acid biosynthesis seems also to be expressed under acidic condition, considering that some of the DEGs were related with cell-wall biogenesis (**Figure 1**). A rerouting of pyruvate metabolism to fatty acid metabolism has been shown for *L. casei, L. delbruecckii* and *L. rhamnosus* under low pH environments (Broadbent et al., 2010; Koponen et al., 2012; Zhai et al., 2014). This metabolic shift aims to reorganize the cell-wall by decreasing both its fluidity and its permeability to protons, which helps to maintain its integrity. In fact, increased levels of long-chained, mono-unsaturated fatty acids in *Streptococcus gordonii, Streptococcus salivarius* and in *L. casei* was observed under acidic environment (Fozo et al., 2004). Specifically, increased proportions of C18:1 and cyclopropane C19:0, with concomitant decreases in C16:0 and cyclopropane C17:0 was observed in *L. casei* stressed by low-pH conditions (Fozo et al., 2004). Our data showed that cell-envelope encoded functions were found associated with DEGs in *L. delbrueckii* (LDB_RS08585), in *L. fermentum (*LAF_RS08425), in *L johnsonii* (LJ_RS04555 and LJ_RS04550), and with some genes of *L. gasseri, L. rhamnosus* and of *L. salivarius* **(Table S3)**, clearly suggesting the importance of cell-wall remodeling for microbial acid tolerance.

Moreover, our data also suggest an important role of protein translation as an adaptive response to acidified environments. Elongation factors, ribosomal proteins, tRNA synthetase, translation initiation factors and transcription termination factors were some of the encoded proteins found in *L. acidophillus, L. brevis, L. casei, L. crispatus, L. delbrueckii, L. fermentum, L. gasseri, L. jensenii, L. johnsonii, L. paracasei, L. plantarum, L. rhamnosus* and in *L. salivarius* (Table S3). An overexpression of genes encoding for the SsrA-binding protein SmpB, such as *L. paracasei* (*smp*B), *L. fermentum* (*LAF_RS02135*), *L. gasseri* (*LGAS_RS06395*), *L. johnsonii* (*LJ_RS03700*) and *L. rhamnosus* (*LGG_RS04515*) was also observed **(Table S3)**. This protein tags proteins whose biosynthesis has stalled or has been interrupted, allowing incomplete-tagged proteins to be

degraded by intracellular proteases. This is an important system responsible for protein quality control (Karzai et al., 2000). Several ribosomal proteins, both from the 30S and from the 50S units, were encoded by *rpsD* gene from *L. casei*, *rplC* from *L. crispatus*, *rpsE* from *L. johnsonii* and by *JDM1_RS04365* from *L. plantarum,* being those genes exhibiting the highest Log2fold change values in those genomes (**Table S4**). Furthermore, an upregulation of genes related to elongation factors that facilitate the translocation of ribosomes on mRNA yielding protein synthesis, such as the *fusA* of *L. rhamnosus*, were also found in this study. Interestingly, some evidences even suggest that in *Escherichia coli* elongation factors act as folding templates for denaturated polypeptides, performing a protein repair compatible-functions under low pH (Caldas et al., 2000), which seems also be the case for *Lactobacilli*. Within protein translation, subunits of H^+-ATP proton pump protein and chaperones (DnaK, GroES) were also associated with overexpressed genes of *L. buchneri* **(Table S4)**.

In this sense, protein repair was also within the biological functions attributed to some of the DEGs. It includes the heat-shock stress proteins, such as trigger factors, chaperones, such as GroEL, GroES and DnaK, and accessory proteins GrpE and DnaJ, in *L. acidophillus, L. brevis, L. buchneri, L. crispatus, L. fermentum, L. gasseri, L. johnsonii, L. paracasei* and *L. rhamnosus* (Table S3). A biological function of Clp protease was also attributed to some the DEGs in *L. crispatus, L. gasseri, L. paracasei* and in *L. salivarius* **(Table S3)**. Wrongly misfolded or damaged proteins due to acidic pH may be either refolded by chaperones or irreversibly removed from the intracellular compartment by the action of proteases (Frees et al., 2007; Papadimitriou et al., 2016). Therefore, both mechanisms contribute to prevent protein structural alterations that could negatively impair microbial metabolism. These findings agree with previous studies showing an overexpression of GroEL and GroER proteins in *L. paracasei*, *L. delbrueckii*, *L. acidophilus* and in *L. plantarum* during acid adaptation (De Angelis & Gobbetti, 2004; Falentin et al., 2010; Wu et al., 2014; Zhang et al., 2020).It was also possible to observe an overexpression of the gene *LSEI_1848* in *L. paracasei* **(Table S3)** that encodes a superoxide dismutase. This finding indicates that distinct stress-related functions may be recruited for the acid tolerance response.

Acid tolerance of *Lactobacillus* spp. seem also to rely on signal transduction systems **(Figure 1)**. In general, two-component signal transduction systems are essential for bacterial survival and adaptation to environmental conditions (Parkinson, 1993). These systems sense modifications and send intracellular signals which induce adaptive changes, especially at gene expression level. Some transduction systems have been reported in *Lactobacilli* spp, as in *L. acidpophilus* and *L. delbrueckii,* being activated at low-pH under *in vitro* conditions (Azcarate-Peril et al., 2005; Cui et al., 2012). Our data, though, showed that signal transduction was upregulated only in *L. curvatus* and in *L. salivarius* (**Table S3**). We hypothesize that the alternated acidic/neutral cycles frequently found under clinical conditions, as those that the carious root surfaces are exposed to, are recruiting many other genes for acid-tolerance than those associated with transduction systems.

DNA-associated functions, such as transposase-type proteins, were also attributed to 31 DEGs, as *L. curvatus* OA78_RS05010, *L. delbrueckii* LDB_RS07740 and *L. fermentum* LAF_RS01020 (**Table S4**). This same mechanism was previously observed by (Broadbent et al., 2010) who hypothesized that mobile DNA elements might be considered as an important microbial evolutionary and adaptive mechanism. In this present study, the role played by these mobile elements in the response to the acidic environment may be suggested, but it needs further clarification. The scientific literature still lacks information regarding the biological processes of many of the DEGs and of those genes that presented the highest Log2fold values. Such invaluable information can infer on other potential processes responsible for an acid tolerance response. Nevertheless, is its clear from our data that the acid tolerance response in *Lactobacillus* spp. involves multiple functions.

We also observed the whole functional activity differs among the assessed genomes **(Table 1)** which was also previously reported (Dame-Teixeira et al., 2020)**.** *L. crispatus, L. fermentum, L. gasseri, L. rhamnosus* and *L. salivarius* presented the highest percentage of DEGs in relation to all genes, ranging from 60.58 to 80.47%, suggesting these genomes were highly active in the carious root lesions samples. Interestingly, these species showed the lowest percentage of acid tolerance DEGs in relation to all DEGs, which could be attributed to the high number of total overexpressed genes found in those genomes. Except for *L. brevis, L. delbrueckii* and *L. jensenii*, which presented a low total number of DEGs, and consequently, a high percentage of acid tolerance DEGs in relation to all DEGs, our

data also indicate that acid tolerance, although an important feature for microbial fitness under a cariogenic environment, might not require much from the *Lactobacillus* physiological machinery. *L. salivarius, L. rhamnosus, L. fermentum, L. gasseri, L. crispatus* and *L. acidophilus* standed out as those showing higher number of acid tolerance DEGs. These differences on functionality, may help to explain the differences on the species-specificity of biological functions attributed to the acid tolerance DEGs (Guan & Liu, 2020; Papadimitriou et al., 2016; Wu et al., 2012, 2013) **(Figure 2).** Instead of exhibiting translation and biogenesis functions, *L. plantarum* and *L. curvatus* exhibited nucleotide transport and metabolism (*L. plantarum*) or nucleotide transport and metabolism, elements of DNA and signal transduction (*L. curvatus*) as the most abundant functions attributed to DEGs. Furthermore, the amino acid transport and metabolism function seemed to be also related to acid tolerance in *L. delbrueckii* and in *L. jensenii* while protein repair seem to be most expressed in *L. buchneri* and in *L. johnsonii*. Energy production function and conversion appeared to be more related to acid tolerance in *L. acidophillus* and in *L. brevis*. It is also important to consider that the variability on mRNA raw counts found in carious root surfaces **(Table S2)** may also be attributed to inherent differences on microbial composition among the participants.

One may be concerned about the lack of microbial abundance data in this study. Although it might be considered as a limitation of the study, we reinforce that any difference on genomes abundance seems irrelevant as samples were assessed at the transcriptional level, and the data of gene expression was normalized accordingly. On contrary, the advantage of this study is that it relied on metatranscriptome analysis, which provides a broad overview of gene expression in clinical samples. To strengthen our findings, though, future research should isolate and study individual strains of *Lactobacillus* spp. from clinical samples under controlled low pH conditions. This approach would offer a more focused and precise assessment of the genes associated with acid tolerance within this specific bacterial group, confirming our results.

Based on the present metatranscriptome data, many biological pathways seem to contribute to acid tolerance response in *Lactobacillus* spp. isolated from biofilm/dentine samples from root carious lesions, being the aciduricity a species-specific trait. While these data help us to understand the physiological and adaptive changes in *Lactobacillus*

resulting from the health-disease process, mechanistic studies must be conducted to validate the genes identified here. The data from this study demonstrate that the response of *Lactobacillus* spp. to an acidic environment is complex and multifaceted. This finding suggests several possible avenues for further research into the adaptive mechanisms of these bacteria.

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Authors contribution

Heitor S. de B. Santos: Investigation, Data curation; Formal analysis; Writting -Original Draft; Visualization; **Nailê Damé-Teixeira:** Conceptualization, Investigation, Data curation; **Martina Hitomi Nagano:** Investigation; Data curation; Visualization; **Thuy Do:** Conceptualization, Investigation, Writing - Review & Editing; **Clarissa Cavalcanti Faturi Parolo:** Conceptualization, Supervision, Writing - Review & Editing; **Marisa Maltz** Conceptualization, Supervision, Writing - Review & Editing; **Rodrigo Alex Arthur:** Conceptualization, Data Curation, Supervision, Visualization, Writting -Original Draft; Writing - Review & Editing

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Figure captions

Figure 1 – Number of acid tolerance DEGs associated with specific biological processes. Numbers above bars represent the percentage of genes in relation to all acid tolerance DEGs (653 genes)

Figure 2 – Biological processes attributed to acid tolerance DEGs in each *Lactobacillus* genome.

Figure 3. Biological processes most frequently attributed to DEGs. Biological processes that are the most or the second most abundant ones in each genome are represented in black and in underline, respectively. Bold followed by asterisk indicates the biological process corresponds to the highest Log2fold change value in that genome.

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Tables

Table 1. Total number of *Lactobacillus* spp. genes, total number of all differently expressed genes (DEGs; % in relation to total number of genes), total number of genes potentially associated with acid tolerance (% in relation to total number of genes), number of acid tolerance-associated DEGs between sound root and carious root surfaces (% in relation to the number of acid tolerance genes) and the percentage of acid tolerance-associated DEGs in relation to all DEGs:

Genome	Total number of genes	Total number of all DEGs	Total number acid tolerance-associated genes	Number of acid tolerance-associated DEGs*	Percentage of acid tolerance-associated DEGs in relation to all DEGs
L. acidophilus	1,832	108 (5.89)	116 (6.33)	33 (28.44)	30.55
L. brevis	2,185	26 (1.18)	103 (4.71)	11 (10.69)	42.30
L. buchneri	2,383	62 (2.60)	117 (4.90)	16 (13.67)	25.80
L. casei	2,765	21 (0.75)	142 (5.13)	1 (0.70)	4.76
L. crispatus	1,934	1,294 (66.9)	96 (4.96)	91 (94.79)	7.03
L. curvatus	1,960	34 (1.73)	107 (5.45)	3 (2.80)	8.82
L. delbrueckii	1,808	23 (1.27)	110 (6.08)	12 (10.90)	52.17
L. fermentum	1,946	1,535 (78.87)	125 (6.42)	98 (78.4)	6.38
L. gasseri	1,772	1,426 (80.47)	108 (6.09)	92 (85.18)	6.45
L. jensenii	1,405	7 (0.49)	95 (6.76)	4 (4.21)	57.14
L. johnsonii	1,804	214 (11.86)	96 (5.32)	41 (42.70)	19.15
L. paracasei	2,764	71 (2.56)	180 (6.51)	17 (9.44)	23.94
L. plantarum	2,883	20 (0.69)	87 (3.01)	4 (9.4.69)	20.0
L. rhamnosus	2,745	1,663 (60.58)	131 (4.77)	114 (87.02)	6.85
L. salivarius	1,876	1,364 (72.70)	124 (6.60)	116 (93.54)	8.50

*All acid tolerance associated DEGs showed Log2fold change >1 indicating an overexpression in carious root surfaces

Supplementary Tables legend

Table S1 – mRNA raw read count attributed to *Lactobacillus* spp. genomes in sound root surfaces

Table S2 – mRNA raw read count attributed to *Lactobacillus* spp. genomes in carious root surfaces

Table S3. Acid tolerance DEGs and the respective biological processes in *Lactobacillus* spp. genome.

Table S4 – Biological processes attributed to acid tolerance DEGs (% of DEGs) and genes showing the highest Log2fold change together with their biological process in each genome