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ORIGINAL ARTICLE

Virulence of *Lactobacillus* spp. misidentified as *Enterococcus faecalis* from children's carious dentine

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ABSTRACT

Objective: This study aimed to search for *Enterococcus faecalis* in children's deep carious dentine and characterize their virulence traits.

Material and Methods: Eight isolates from 15 carious molars identified by 16S rDNA species-specific PCR as *E. faecalis* were included. These eight isolates were subject to identification by MALDI-TOF and characterized regarding: (i) bacterial aggregation and biofilm formation on polystyrene and glass, with/without saliva, as single or dual-species (associated to *Streptococcus mutans*); (ii) environmental pH measurement before and after 24 h incubation; (iii) acidogenicity; (iv) gelatinase production; (v) macrophage adherence; and (vi) toxicity towards *Caenorhabditis elegans*. Statistical analyses were performed using two-way ANOVA/Tukey or Fisher's exact tests.

Results: All isolates initially identified as *E. faecalis* by PCR were correctly identified as *Lactobacillus* by MALDI-TOF, being designated as *Lactobacillus* misidentified as *Enterococcus* (LME). These isolates produced biofilm in the presence of saliva and in the dual-species assays. Bacterial aggregation was only observed in the dual-species model. After 24 h, environmental pH dropped from 7.5 to 4.5 for seven of eight isolates, and to 4.0 in all dual-species models. LME isolates were acidogenic, none of them produced gelatinase or adhered to macrophages, but all presented toxicity towards *C. elegans*.

Conclusions: No *E. faecalis* were identified in the children's caries lesions. All LME isolates presented important virulence traits, including biofilm formation and high acidogenicity, which cause enamel demineralization, that might increase the risk of dental caries in children carrying LME. Thus, the correct identification and in-depth virulence characterization of microorganisms isolated from dental caries are important to understand the dynamics of this disease.

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Introduction

Dental caries can be determined by biological, behavioural, psychosocial and environmental factors [1]. This disease results from acid production by a complex oral microbiota in the presence of a carbohydrate-frequent diet leading to an acidic environment and consequent demineralization of tooth enamel [2]. The composition of the oral microbiota has been extensively studied [2–6] and several microorganisms have been associated with the cariogenic process, including *Streptococcus mutans*, *Veillonella*, *Lactobacillus*, *Bifidobacterium*, *Cutibacterium*, low-pH non-*S. mutans* streptococci, *Actinomyces* and *Atopobium* [3]. Furthermore, higher levels of *Campylobacter*, *Streptococcus*, *Lactobacillus* and *Prevotella* are related to an increased risk of dental caries [6].

The composition of the carious microbiota varies as dentine caries progresses and deep dentinal caries lesions seem to be one of the primary sources of bacteria for endodontic infections [5]. The species *Enterococcus faecalis* has been described as prevalent in these infections in primary teeth [7,8] and more related to the oral microbiota of caries-active than caries-free children [9].

Although studies have demonstrated the microbial importance in the cariogenic process [4–6], little is known about the dynamics of how these microorganisms interact in the progression of dental caries. Considering the prevalence of *E. faecalis* in endodontic infections and the association of such infections with deep caries lesions, the aim of this study is to search for *E. faecalis* in children's deep carious dentine and characterize their virulence traits.

Materials and methods

Ethical aspects

After approval by the local Research Ethics Committee (protocol number: 1.177.743), each child's parents or legal

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guardians signed a written informed consent form allowing the children to participate in the study.

Human saliva used in this study was collected after volunteers signed a written informed consent form also approved by the local Research Ethics Committee (protocol number: 2.402.672).

Participants

From June 2016 to March 2017, 14 children attending the dental clinic of a Brazilian public university were enrolled in the study. Each child contributed with one sample of deep carious dentine from one molar, except one, who contributed with two samples from two different molars, totalizing 15 samples. All dentine samples were obtained from primary teeth, except one. Participants included six boys and eight girls, aged 4–11 years, and two of them were siblings.

All participants presented deep cavities extending to the inner half of dentine, confirmed by radiographic exam and without pulp involvement. The exclusion criteria were children who: did not cooperate during treatment; had syndromes or chronic systemic diseases; had used antibiotics within the previous 3 months; and presented painful symptoms consistent with irreversible pulpitis or mobility. Dentine samples from teeth with exposure of the dental pulp or periodontal changes, with pathological resorption of roots, or in an advanced stage of physiological resorption (not exceeding 1/3) were also excluded.

Clinical examination was performed by the same dentist with experience in cariology (gold standard) (LAA). The dmft (decayed, missing and filled primary teeth) and DMFT (decayed, missing and filled permanent teeth) indexes [10], and dentine characteristics (colour, consistency and moisture) [11] were evaluated for all children during clinical examination.

Collection and processing of dentine samples

Dentine samples were collected and processed according to Ornellas et al. [12] following serial dilution on triplicate Enterococcosel agar (Becton, Dickinson and Company, Sparks, MD), a selective medium for detection and enumeration of *Enterococcus* genus.

Bacterial control strains

E. faecalis American Type Culture Collection (ATCC) 29212, *Lactobacillus casei* ATCC 393, *Lactobacillus rhamnosus* ATCC 7969, *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* ATCC 12228 and *S. mutans* ATCC 25175 were used as control strains.

Identification of the isolates

Colonies with black zones grown on Enterococcosel agar were subject to Gram staining, catalase and esculin hydrolysis tests [13]. Isolates presumptively identified as *Enterococcus* spp. by these tests were subject to *E. faecalis*-specific PCR [14]. Those identified by this PCR as *E. faecalis* were further characterized by the assays described below and then submitted to identification by Matrix-assisted Laser Desorption Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS) (Bruker Daltonics GmbH, Bremen, Germany) [15], using FlexControl software (version 3.4; Bruker Daltonics GmbH, Bremen, Germany) to compare/cross the data with MALDI Biotyper database (version 3.1; Bruker Daltonics GmbH, Bremen, Germany).

'In vitro' biofilm and aggregation assays

Saliva collection and processing

Whole, unstimulated saliva was collected in sterile tubes from three healthy adults at least 1.5 h after food/drink/toothcleaning, with no antibiotic use within the previous 3 months. Saliva was then pooled and centrifuged (30 min, 4 °C, 2400×*g*). Supernatant was transferred to another sterile tube, pasteurized (65 °C, 30 min) and re-centrifuged under the same conditions. Final supernatant was dispensed into another sterile tube and stored at −4 °C. Efficacy of pasteurization was assessed by inoculation, in triplicate, of 100 μL of saliva, onto brain heart infusion (BHI) agar and broth (Becton, Dickinson and Company, Sparks, MD) and observation of no microbial growth after incubation at 36 °C for 72 h.

Single- and dual-species polystyrene microplate biofilm assay

This quantitative assay was performed in 96-well polystyrene flat bottom microplates according to Valdez et al. [16] with modifications. Isolates were grown on BHI agar for 24 h and then transferred to glass tubes containing BHI broth with 1% sucrose until reaching optical density (O.D.) of 0.5 at 550 nm. Isolates were evaluated alone (single-species) and associated to *S. mutans* ATCC 25175 (dual-species at equal volumes), with final well volume of 200 μL.

Both situations (single- and dual-species) were evaluated in the absence and after previous well coating with saliva. For this, 50 μL of pasteurized saliva were deposited into wells and shaken for 4 h (34 rpm) (Benfer, São Paulo, Brazil) at room temperature. Then, saliva was removed from all wells and microplates were prepared as described above.

After incubation at 36 °C and microaerophilic conditions for 24 h, biofilm was dyed according to Valdez et al. [16], resolubilized with 200 μL of ethanol and evaluated with a Microplate Spectrophotometer (BioTek Epoch, Winooski, VT) at 570 nm. Each isolate was classified as strong (+++), moderate (++), weak (+) and non-producer of biofilm (−) [17]. Each experiment was performed in triplicate at least twice.

Single- and dual-species aggregation and glass tube biofilm assays

To assess qualitative aggregation and biofilm formation on glass surfaces, bacterial suspensions (O.D. 0.1 at 550 nm) in BHI broth supplemented with 1% sucrose were prepared in glass tubes from a 24 h culture on BHI agar. Four sets of

tubes were prepared (final volume 500 μL) to evaluate the isolates on their own (single-species), associated to *S. mutans* (dual-species; at equal volumes), and with addition of pasteurized saliva (50 μL) in both situations (single and dual-species). The four sets of tubes were incubated at 36 °C for 24 h.

Then, bacterial aggregation and adherence were classified according to Murchison et al. [18] with modifications. Tubes were quickly vortexed (5 s) to remove cells that grew close but did not adhere to the glass tubes. Culture fluid was scored to assess aggregation: (–), no visible aggregation; (+), slightly visible minute clumps of cells in turbid fluid; (++), easily visible small clumps of cells in turbid fluid; (+++), well-defined large clumps of cells in clear supernatant fluid. After assessing aggregation, adherence was evaluated as follows: culture fluid was removed from tubes and 500 μL of 1% crystal violet solution was added to each tube for a 5 min period. Tubes were then washed three times with running tap water and let dry for 24 h. Adherence was scored as follows: (–), no visible adherence; (+), few visible cells adhering to bottom of tube; (++), thin confluent coat of cells on bottom of tube; (+++), thick confluent coat of cells on bottom of tube. Each experiment was performed at least two times.

Culture pH measurements

Before dying the biofilm (section 'Single- and dual-species polystyrene microplate biofilm assay'), the content of each triplicate of wells was pooled and subject to pH measurement.

Acidogenicity assay

Each isolate was cultured in 10 mL of BHI broth for 18 h at 36 °C in a sterile 15 mL polypropylene tube. The tubes were then centrifuged (2400×*g* for 10 min at 17 °C) and the pellets resuspended in 2 mL 50 mM KCl and 1 mM $MgCl_2$ (pH 7.0), until reaching an O.D. of 2.0 at 550 nm, and centrifuged (3700×*g* for 10 min at 17 °C). The pellets were resuspended in 2 mL 1× phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 1.8 mM KH_2PO_4; pH 7.4) and incubated for 1 h at 36 °C for starvation, then were centrifuged (3700×*g* for 10 min at 17 °C) and the pellets resuspended in 2 mL 50 mM KCl and 1 mM $MgCl_2$ (pH 7.0). After centrifugation (3700×*g* for 10 min at 17 °C), the pellets were resuspended in 1 mL 50 mM KCl and 1 mM $MgCl_2$ with glucose 55.5 mM adjusted to pH 7.0. The pH of the suspension was recorded immediately and 5, 15, 30, 60, 120, 180 and 240 min after resuspension. The area under the curve (AUC), in cm^2, of each isolate was calculated three times by a single operator using ImageJ 1.52a software (National Institutes of Health, Bethesda, MD, a freeware available at https://imagej.nih.gov). This experiment was performed once in duplicate.

Gelatine liquefaction assay

Gelatine liquefaction assay was performed according to MacFaddin [13], inoculating isolates in tubes containing Todd medium (Becton, Dickinson and Company, Sparks, MD) with 4% gelatine, for 30 days (36 °C) to detect gelatinase producers. This assay was performed at least twice.

U 937 macrophage assays

The U 937 macrophage cell line was cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO) with heat-inactivated 10% foetal bovine serum (FBS) and penicillin 100 U, streptomycin 100 μg/mL, amphotericin B 25 μg/mL. These macrophages were seeded onto plates containing round glass coverslips at a density of 5×10^5 cells/well. Plates were, then, incubated in a humidified atmosphere with 5% CO_2 at 37 °C in DMEM supplemented with 10% FBS and 2 mmol/L L-glutamine for 48 h prior to infection, allowing formation of a confluent monolayer of cells. Bacterial isolates were grown in BHI broth for 24 h/36 °C followed by centrifugation 2400×*g*/10 min and resuspension in 1× PBS. The pellets were washed three times and resuspended in 1× PBS. Final resuspensions were in antimicrobial-free-DMEM and were adjusted to O.D. 0.2 at 680 nm. Cultured macrophages were washed three times with 1× PBS. To determine the adhesion pattern, the cells were infected for 90 min at a MOI of 100:1 at 37 °C in 5% CO_2 and washed six times with 1× PBS. Briefly, cells are pre-fixed with 4% w/v of formaldehyde for 1 h at room temperature, washed and fixed with absolute methanol. Once again wells were washed three times with 1× PBS and stained with Giemsa at room temperature. The coverslips were then washed with distilled water, dried at room temperature, mounted on glass slides and examined under optical microscopy (200× magnification).

To evaluate survival of isolates in U 937 macrophages, Coburn et al. [19] methodology was chosen, using gentamicin (250 μg/mL) or streptomycin (100 μg/mL)–penicillin (100 U), to eliminate non-internalized bacteria.

Caenorhabditis elegans *infection model*

C. elegans N2 wild-type was maintained on nematode growth medium (NGM) agar (in house) inoculated with *Escherichia coli* OP50 for seven days until the worms became starved. Isolates were grown confluently on NGM agar (36 °C and microaerophilic conditions) 48 h prior to infection. Approximately, seven synchronous L4 worms were transferred to each inoculated plate, incubated at room temperature for five days and survival was scored daily. Worms were considered dead when they did not respond to gentle mechanical stimulation. Toxicity towards *C. elegans* was classified according to their survival rates: nontoxic (–), more than 50% alive in 120 h; weak (+), more than 50% died in 120 h; moderate (++), all worms died in 96 h; and strong (+++), all worms died in 48 h.

Statistical analysis

Data were statistically analysed using GraphPad Prism (version 6.0, La Jolla, CA) software, with 95% level of confidence.

Comparative analyses between the results from the clinical data, regarding age, dmft+DMFT and colour/consistency/moisture of dentine samples ($n = 8$), were performed by Fisher's exact test. To assess the statistical significance of the differences between acidogenic abilities of the isolates ($n = 8$ plus three control samples, totalling 11 bacterial samples) and their toxicity towards *C. elegans* ($n = 8$ plus 1 control sample, totalling nine bacterial samples), two-way ANOVA followed by Tukey's test were used. Statistical significance was considered when p values $<.05$ were obtained.

Results

Presence of colonies with black zones on Enterococcosel agar was observed for nine (60%) of the 15 samples. Eight (88.9%) of these nine, were Gram-positive cocci, catalase negative and esculin positive, being presumptively identified as *Enterococcus* genus and were initially identified as *E. faecalis* by PCR.

MALDI-TOF MS identified these eight isolates as: *Lactobacillus paracasei* (four isolates), *L. rhamnosus* (three isolates) and *Lactobacillus plantarum* (one isolate) (Table 1). They were identified with score values higher than 2.000, which represents secure genus and probable species identification. These eight isolates misidentified as *E. faecalis* were, then, designated as *Lactobacillus* misidentified as *Enterococcus* (LME). At this point, Gram staining was repeated for all LME isolates and Gram-positive rods were observed.

Samples containing these isolates were collected from seven children, including two siblings and one child who contributed with two dentine samples. The mean age of the children was 7.4 ± 1.8. Even though not statistically significant, those with LME in their carious dentine were younger (6.6 ± 1.5) than those with no LME (8.1 ± 1.9) ($p = .13$). In relation to dmft+DMFT index, the mean was 4.9 ± 1.6 and 3.9 ± 2.7 ($p = .62$), for the children with and without LME, respectively (Table 1).

In regard to dentine characteristics all were brown; 12 rigid and three non-rigid; 10 wet and five dry. No statistical differences were observed between dentine containing LME and those without LME ($p = .28$). Both siblings had brown, non-rigid and wet dentins.

Table 2 shows the results of different assays to detect biofilm production and aggregation of each LME isolate separately, as well as in association with saliva and/or a type strain known for its biofilm production ability (*S. mutans* ATCC 25175).

The polystyrene microplate biofilm assay detected biofilm production for seven (87.5%) LME isolates. In the presence of saliva, all LME were biofilm producers. In contrast, only one isolate produced biofilm on glass, regardless of the presence of saliva. In all the dual-species assays, biofilm formation was observed, so biofilm production by *S. mutans* was not affected by the presence of LME isolates (Table 2).

Bacterial aggregation was only observed in the dual-species assays, minimally affecting *S. mutans'* single-species aggregation pattern.

After incubation for 24 h at 36 °C and microaerophilic conditions, pH values dropped from 7.5 to 4.5 for all, but one (final pH = 5.0), LME isolate. In the dual-species assays, pH values decreased from pH 7.5 to 4.0 for all LME (Table 1).

Table 1. Description of the participants and characterization of LME isolates.

Isolate	Teeth	Age	dmft + DMFT	PCR identification	MALDI-TOF identification	Biofilm[A]	Medium pH after 24 h[B]				AUC (cm^2)[C]	Gelatinase[D]	Macrophage adherence	Toxicity to *C. elegans*[E]
							I	I + Sl	I + S.m	I + S.m + Sl				
LME isolates														
1*	74	7	3	*E. faecalis*	*L. rhamnosus*	+	4.5	4.5	4.0	4.0	20.74a	–	Non-adherent	(+++)
2	65	6	6	*E. faecalis*	*L. paracasei*	+	4.5	4.5	4.0	4.0	29.36a	–	Non-adherent	(++)
3	84	6	7	*E. faecalis*	*L. rhamnosus*	+	4.5	4.5	4.0	4.0	28.20a	–	Non-adherent	(+)
4	55	7	5	*E. faecalis*	*L. plantarum*	–	5.0	5.0	4.0	4.0	35.78a,b	–	Non-adherent	(++)
5*	84	4	6	*E. faecalis*	*L. rhamnosus*	+++	4.5	4.5	4.0	4.0	48.63a,b	–	Non-adherent	(+++)
6	84	7	4	*E. faecalis*	*L. paracasei*	+	4.5	4.5	4.0	4.0	28.00a	–	Non-adherent	(++)
7**	65	9	3	*E. faecalis*	*L. paracasei*	+	4.5	4.5	4.0	4.0	35.89a,b	–	Non-adherent	(+++)
8**	75			*E. faecalis*	*L. paracasei*	+	4.5	4.5	4.0	4.0	29.58a	–	Non-adherent	(+++)
Mean		6.6 ± 1.5	4.9 ± 1.6								32 ± 8.2			
Control strains														
L.r	na	na	na	/	/	+	4.5	4.5	4.0	4.0	24.15a	/	Non-adherent	(+++)
L.c	na	na	na	/	/	+	4.5	4.5	4.0	4.0	24.08a	/	/	/
S.m	na	na	na	/	/	+++	4.0	4.0	na	na	63.67b	/	/	/
S.a	na	na	na	/	/	/	/	/	/	/	/	+	/	/
S.e	na	na	na	/	/	/	/	/	/	/	/	(+)	/	/
E.f	na	na	na	/	/	/	/	/	/	/	/	(+)	/	/

dmft: decayed-missing-filled teeth in the primary dentition; DMFT: decayed-missing-filled teeth in the permanent dentition; PCR: polymerase chain reaction; AUC: area under the curve – the AUC was calculated from the pH drop curve, considering pH 2.8 as a cut-off point, expressing the acidogenicity of each sample; LME: *Lactobacillus* misidentified as *Enterococcus*; L.r: *L. rhamnosus* ATCC 7969; L.c: *L. casei* ATCC 393; S.m: *S. mutans* ATCC 25175; S.a: *S. aureus* ATCC 25923; S.e: *S. epidermidis* ATCC 12228; E.f: *E. faecalis* ATCC 29212; na: not applicable; /: not determined.

*Siblings.

**Same child.

APolystyrene microplate biofilm assay: –, absence; +, weak; and +++, strong biofilm production.

Bl: isolate; Sl: saliva; initial medium pH 7.5.

CDifferent lowercase letters in the same column represent statistically different values ($p < .05$).

D–, non-producers; +, producers; and (+), slow producers of gelatinase.

E(+) weak (more than 50% died in 120 h); (++) moderate (all worms died in 96 h); (+++) and strong (all worms died in 48 h) toxicity towards *C. elegans*.

Table 2. Biofilm formation on different surfaces and bacterial aggregation.

	Polystyrene microplate biofilm assay[A]				Glass tube biofilm assay[B]				Bacterial aggregation[C]			
Isolates	I	I + Sl	I + S.m	I + Sl + S.m	I	I + Sl	I + S.m	I + Sl + S.m	I	I + Sl	I + S.m	I + Sl + S.m
1*	+	+	+++	+++	–	–	+++	+++	–	–	++	++
2	+	+	+++	+++	–	–	+++	+++	–	–	++	+++
3	+	+	+++	+++	–	–	+++	+++	–	–	++	++
4	–	+	+++	+++	–	–	+++	+++	–	–	+++	+++
5*	+++	++	+++	+++	+++	+	+++	+++	–	–	++	++
6	+	+	+++	+++	–	–	++	+++	–	–	++	++
7**	+	+	+++	+++	–	–	+++	+++	–	–	++	++
8**	+	+	+++	+++	–	–	+++	+++	–	–	+++	++
L.r	+	+	+++	+++	–	–	+++	+++	–	–	++	++
L.c	+	+	+++	+++	–	–	+++	+++	–	–	++	++
S.m	+++	+++	na	na	+++	+++	na	na	++	+++	na	na

I: isolate; Sl: saliva; L.r: *L. rhamnosus* ATCC 7969; L.c: *L. casei* ATCC 393; S.m: *S. mutans* ATCC 25175; na: not applicable.
*Siblings.
**Same child.
A–, absence; +, weak; ++, moderate; and +++, strong biofilm production.
B–, no visible adherence; +, few visible cells adhering to bottom of tube; ++, thin confluent coat of cells on bottom of tube; +++, thick confluent coat of cells on bottom of tube.
C–, no visible aggregation; +, slightly visible minute clumps of cells in turbid fluid; ++, easily visible small clumps of cells in turbid fluid; +++, well-defined large clumps of cells in clear supernatant fluid.

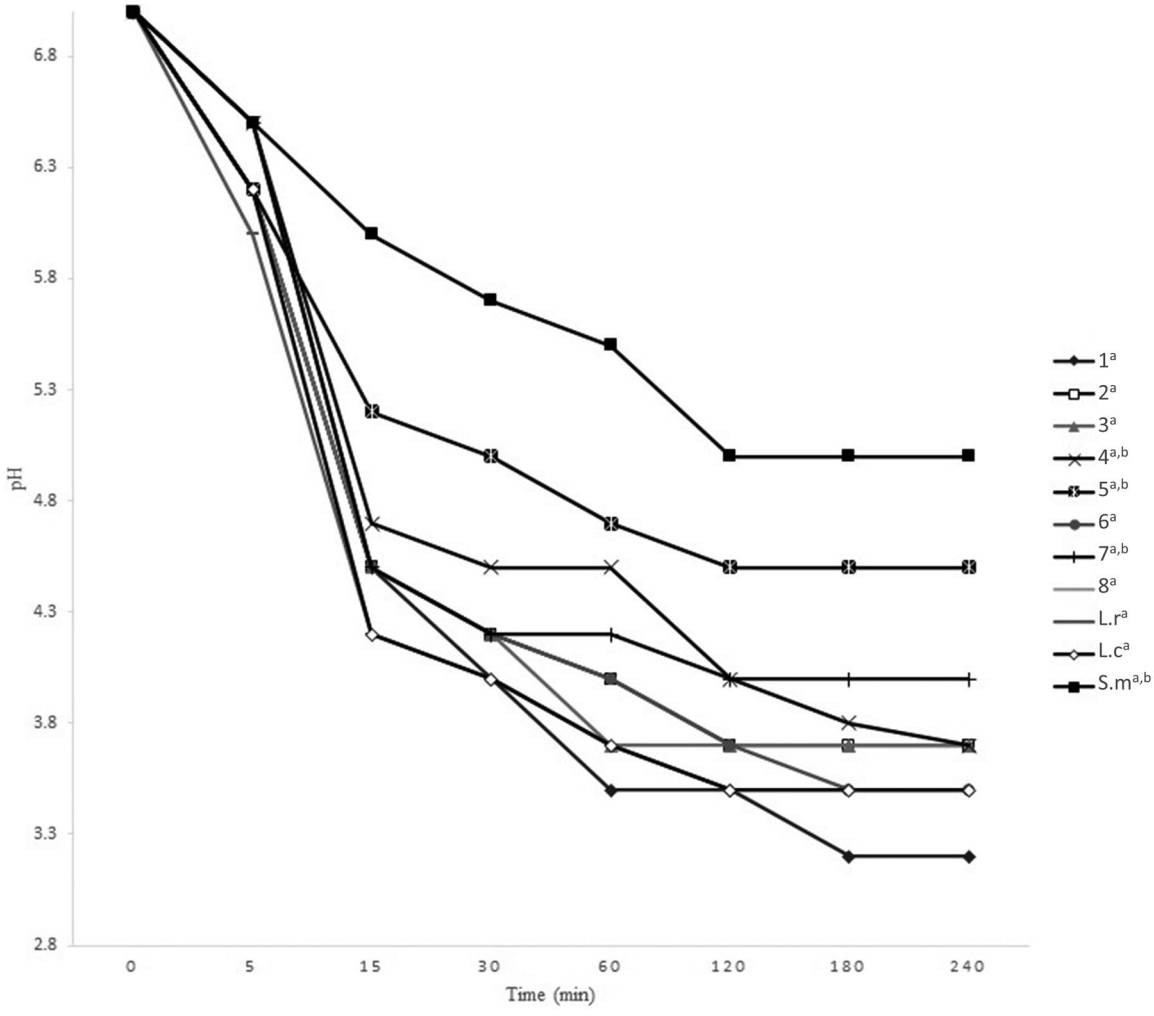

Figure 1. Mean pH values of LME isolates in glucose solution after 240 min. 1–8: LME isolates 1–8; L.r: *L. rhamnosus* ATCC 7969; L.c: *L. casei* ATCC 393; S.m: *S. mutans* ATCC 25175; different lowercase letters represent statistically different values ($p < .05$).

The LME isolates' AUC mean was $32 \pm 8.2\,cm^2$ (range, 20.74–48.63). All isolates were more acidogenic than *S. mutans*, characterized by lower AUC values. For five isolates (isolates 1, 2, 3, 6 and 8), this difference was statistically significant ($p < .05$). No statistically significant differences were observed when comparing the AUC values amongst the LME isolates (Figure 1).

None of the LME isolates produced gelatinase or adhered to U 937 macrophages. Survival in macrophage assay was

Table 3. Survival rate of *C. elegans* infected with LME isolates.

	Time				
Isolate	24 h	48 h	72 h	96 h	120 h
1*	20[a]	0[a]	–[a]	–[a]	–[a]
2	33[a,b]	33[a]	33[a]	0[a]	–[a]
3	100[b]	100[b]	50[a]	25[a]	25[a]
4	86[a,b]	14[a]	14[a]	0[a]	–[a]
5*	50[a,b]	0[a]	–[a]	–[a]	–[a]
6	33[a,b]	17[a]	17[a]	0[a]	–[a]
7**	57[a,b]	0[a]	–[a]	–[a]	–[a]
8**	40[a,b]	0[a]	–[a]	–[a]	–[a]
L.r	67[a,b]	0[a]	–[a]	–[a]	–[a]

L.r: *Lactobacillus rhamnosus* (ATCC 7969); –: same result as previous time period. Approximately, seven worms were used per isolate. The survival rate expresses the percentage of worms alive at each time. Different lowercase letters in the same column represent statistically different values ($p < .05$).
*Siblings.
**Same child.

not performed since all isolates were resistant to gentamicin and penicillin–streptomycin.

In the *C. elegans* toxicity assay, at day 1, *C. elegans* infected by isolate 3 had statistically higher survival rate than the ones infected by isolate 1 ($p < .05$). At day 2, the survival rate of *C. elegans* infected by isolate 3 remained stable, with statistical difference to all other isolates ($p < .05$). Isolates 1, 5, 7, 8 and the *L. rhamnosus* control strain presented strong toxicity to *C. elegans*, while toxicity of isolates 2, 4 and 6 was moderate and of isolate 3 was weak (Table 3).

Discussion

Several studies on the microbiological aspects of dental caries have been conducted with children and adults [3,4,20–22]. None of them, however, indicated the presence of *E. faecalis* in carious dentine, except for one study of root caries in elderly patients [20]. In contrast, some genus, such as *Lactobacillus*, have been reported as more abundant in deeper layers of caries lesions [5].

In this study, eight *Lactobacillus* isolates from children's carious dentine were initially misidentified as *E. faecalis*. The growth of these isolates in selective medium was the first step towards misidentification. The second one was the morphological characteristics observed after Gram staining and the third was the results of the 16S rDNA *E. faecalis*-specific PCR.

Several *Lactobacillus* species are bile tolerant and grow in the presence of oxgall [23], a component of the Enterococcosel agar that inhibits the growth of Gram-positive bacteria other than enterococci. This might explain LME isolates' growth on this selective medium. *Enterococcus* and *Lactobacillus* [24,25] share several characteristics, but a key feature to distinguish them is the cellular morphology on Gram staining. Here, Gram staining performed directly from primary culture on selective *Enterococcus* medium and under non-ideal environmental conditions could help explain the initial misidentification. *Lactobacillus* appearing as Gram-positive cocci had already been previously described [26].

An 'in silico' analysis of the primer pair used in this study by Nucleotide Blast free tool (https://blast.ncbi.nlm.nih.gov/), with no algorithm parameter alterations, showed only *E. faecalis* species amplification. However, when maximum target sequences were expanded and *Enterococcus* was excluded from the search, possible matches included *Enterobacter*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Staphylococcus* and *Weissella* (data not shown). These primers demonstrated lack of specificity in detecting *E. faecalis*, demonstrating the importance of performing a detailed 'in silico' primer pair analysis prior to use. Some of the microorganisms that can be amplified with this primer pair are associated with endopathogenic microbiota [27–29]. Hence, previous studies [14,30,31] that used the same PCR primers to detect *E. faecalis* in endodontic samples could have misidentified this species.

Isolates were properly identified as *Lactobacillus* spp. only by MALDI-TOF MS, which consists of a rapid and reliable approach for microbial identification [32] and indicates presumptive species and correct genus identification for *Lactobacillus* [15]. The change in morphology observed in the second Gram staining might be due to the fact that it was performed with the samples grown on appropriate medium and environmental conditions.

Dental caries is a complex, multifactorial, polymicrobial and biofilm-induced disease resulting in loss of tooth hard tissues [16,33]. Here, two tests were performed to assess biofilm production, a quantitative assay on polystyrene surface and a qualitative one on glass surface. Different results were observed, especially for the LME single-species biofilm. This could have been due to the two different surface materials used in these assays and/or because the qualitative method was read macroscopically (less sensitive) and the quantitative method was read by an equipment that quantifies the absorbance, being, therefore, more sensitive.

Bowen [34] highlights the importance of saliva and sucrose in 'in vitro' biofilm assays to resemble the matrix structure of oral biofilms found in humans. Accordingly, in the present study, sucrose was the carbohydrate of choice to supplement the medium of the biofilm assays, and the tests were performed in the presence and absence of saliva. All LME isolates produced biofilm, an important characteristic in the cariogenic process, by the quantitative method on the wells previously coated with saliva.

S. mutans adheres to enamel in caries initiation enabling the binding of other species, such as *Lactobacillus*, forming a more complex aciduric and acidogenic biofilm, and is, therefore, a risk factor for caries progression [2,22]. Here, LME, isolated from carious dentine, allowed stronger biofilm production by *S. mutans* in the presence of saliva in both assays, showing that the dual-species biofilm with all LME isolates presented this important cariogenic trait. When a dual-species biofilm assay was performed with LME isolates and *Streptococcus salivarius* ATCC 13419, instead of *S. mutans*, biofilm formation was also observed (data not shown).

According to Kim et al. [35], the acidogenic ability of dental biofilms is a significant predictor for caries occurrence. This ability could be observed for all LME isolates as they presented low AUC values, indicating high acidogenicity. Studies have suggested that microorganisms with potential

to be oral probiotics are able to: produce bioactive substances such as bacteriocins; inhibit the growth, co-aggregation, biofilm formation and gene expression of caries-inducing *S. mutans*; and increase environmental pH [36–38]. All LME isolates did not decrease the *S. mutans* biofilm nor increase environmental pH. Additionally, these isolates had high acidogenic ability and maintained similar *S. mutans* aggregation patterns. Although some *Lactobacillus* spp. have been proposed as promising oral probiotics [37,38], we strongly believe that LME isolates were, instead, important in the cariogenic process. In 2014, Schwendicke et al. [39], warned about the possibility of a probiotic strain of *L. rhamnosus* increasing the cariogenic potential of *S. mutans* by the induction of mineral loss, especially in dentine cavities and under high cariogenic conditions [39].

Bacterial adherence to macrophages is considered a virulence characteristic that can be involved in colonization as well as in establishing metastatic foci of infection [40]. Our results showed that LME isolates were non-adherent to U 937 macrophages. So far, this ability had not been evaluated for *Lactobacillus* genus. It was not possible to perform the survival in macrophage assay since all isolates were resistant to gentamicin and penicillin–streptomycin. Of note, all LME were resistant to antimicrobial agents commonly used in the treatment of endocarditis by *Lactobacillus* (penicillin associated to an aminoglycoside) [41].

C. elegans is a useful model to observe host–microbe interactions and virulence traits of a variety of pathogens. Studies diverge on *C. elegans*' response to lactic acid bacteria, and the differences observed seem to be strain-dependent [42,43]. Toxicity of the LME isolates towards *C. elegans* varied, and most isolates were moderately or highly toxic. The four highly toxic LME isolates were the ones recovered from siblings and the child who provided two samples.

Recently, it was revealed by multilocus sequence typing unambiguously identical *Lactobacillus fermentum* strains in the saliva of children in the same classroom, suggesting a potential involvement of saliva as a vehicle for transmission of oral microorganisms between these children [44]. In this study, the siblings both had LME isolates and dentine with similar characteristics. This could suggest a possible transmission of these microorganisms among family members, as previously suggested for *S. mutans* [45]. However, genotypic tests such as multilocus sequence typing or pulsed-field gel electrophoresis should be performed to investigate this possibility.

Despite the clear relationship between deep caries lesions and endodontic infections, this study did not detect *E. faecalis* in children's deep carious dentine. To the best of our knowledge, no previous study performed such extensive virulence characterization of *Lactobacillus* spp. isolates from deep carious dentine. This study was the first to describe *Lactobacillus* initially misidentified by PCR as *E. faecalis*. Even though *Lactobacillus* spp. are frequently reported as promising probiotics, these LME isolates had important virulence traits known to be associated with caries, including biofilm formation and high acidogenicity. Characteristics that could cause complications in case of metastatic infections, such as biofilm production and resistance to antimicrobial agents commonly used for treatment of *Lactobacillus* infections, were also observed. In several aspects, the isolates were different from each other, but similar characteristics were observed when comparing isolates from siblings. Our findings indicate the importance of in-depth characterization of microorganisms isolated from dental caries in order to further understand the dynamics of this disease. Further studies are needed to compare and understand the virulence characteristics of non-LME *Lactobacillus* and LME isolated from carious dentine.

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Disclosure statement

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References

[1] Machiulskiene V, Campus G, Carvalho JC, et al. Terminology of dental caries and dental caries management: consensus report of a workshop organized by ORCA and cariology research group of IADR. Caries Res. 2020;54(1):7–14.
[2] Tanner ACR, Kressirer CA, Rothmiller S, et al. The caries microbiome: implications for reversing dysbiosis. Adv Dent Res. 2018;29(1):78–85.
[3] Aas JA, Griffen AL, Dardis SR, et al. Bacteria of dental caries in primary and permanent teeth in children and young adults. J Clin Microbiol. 2008;46(4):1407–1417.
[4] Xu H, Hao W, Zhou Q, et al. Plaque bacterial microbiome diversity in children younger than 30 months with or without caries prior to eruption of second primary molars. PLOS One. 2014;9(2):e89269.
[5] Rôças IN, Alves FR, Rachid CT, et al. Microbiome of deep dentinal caries lesions in teeth with symptomatic irreversible pulpitis. PLOS One. 2016;11(5):e0154653.
[6] Kazemtabrizi A, Haddadi A, Shavandi M, et al. Metagenomic investigation of bacteria associated with dental lesions: a cross-sectional study. Med Oral Patol Oral Cir Bucal. 2020;25(2):e240–e251.

[7] Cogulu D, Uzel A, Oncag O, et al. PCR-based identification of selected pathogens associated with endodontic infections in deciduous and permanent teeth. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2008;106(3):443–449.
[8] Cancio V, Carvalho Ferreira DD, Cavalcante FS, et al. Can the *Enterococcus faecalis* identified in the root canals of primary teeth be a cause of failure of endodontic treatment? Acta Odontol Scand. 2017;75(6):423–428.
[9] Kouidhi B, Zmantar T, Mahdouani K, et al. Antibiotic resistance and adhesion properties of oral Enterococci associated to dental caries. BMC Microbiol. 2011;11(1):155.
[10] Petersen PE, Baez RJ, World Health Organization. Oral health surveys: basic methods. 5th ed. Geneva: WHO Library Cataloguing-in-Publication Data; 2013. Chapter 3.2, Post-survey assistance; p. 73–76.
[11] Kidd EAM, Joyston-Bechal S, Beighton D. Microbiological validation of assessments of caries activity during cavity preparation. Caries Res. 1993;27(5):402–408.
[12] Ornellas PO, Antunes LS, Motta PC, et al. Antimicrobial photodynamic therapy as an adjunct for clinical partial removal of deciduous carious tissue: a minimally invasive approach. Photochem Photobiol. 2018;94(6):1240–1248.
[13] MacFaddin JF. Biochemical tests for identification of medical bacteria. Philadelphia (PA): Williams and Wilkins; 1977.
[14] Siqueira JF Jr., Rôças IN. Polymerase chain reaction-based analysis of microorganisms associated with failed endodontic treatment. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2004;97(1):85–94.
[15] Dec M, Puchalski A, Urban-Chmiel R, et al. 16S-ARDRA and MALDI-TOF mass spectrometry as tools for identification of *Lactobacillus* bacteria isolated from poultry. BMC Microbiol. 2016;16(1):105.
[16] Valdez RMA, Dos Santos VR, Caiaffa KS, et al. Comparative in vitro investigation of the cariogenic potential of bifidobacteria. Arch Oral Biol. 2016;71:97–103.
[17] Stepanović S, Vuković D, Dakić I, et al. A modified microtiter-plate test for quantification of staphylococcal biofilm formation. J Microbiol Methods. 2000;40(2):175–179.
[18] Murchison H, Larrimore S, Curtiss R. Isolation and characterization of *Streptococcus mutans* mutants defective in adherence and aggregation. Infect Immun. 1981;34(3):1044–1055.
[19] Coburn PS, Baghdayan AS, Dolan GT, et al. An AraC-type transcriptional regulator encoded on the *Enterococcus faecalis* pathogenicity island contributes to pathogenesis and intracellular macrophage survival. Infect Immun. 2008;76(12):5668–5676.
[20] Preza D, Olsen I, Aas JA, et al. Bacterial profiles of root caries in elderly patients. J Clin Microbiol. 2008;46(6):2015–2021.
[21] Eriksson L, Holgerson PL, Johansson I. Saliva and tooth biofilm bacterial microbiota in adolescents in a low caries community. Sci Rep. 2017;7(1):1–12.
[22] Hurley E, Barrett MP, Kinirons M, et al. Comparison of the salivary and dentinal microbiome of children with severe-early childhood caries to the salivary microbiome of caries-free children. BMC Oral Health. 2019;19(1):13.
[23] Hu PL, Yuan YH, Yue TL, et al. A new method for the in vitro determination of the bile tolerance of potentially probiotic lactobacilli. Appl Microbiol Biotechnol. 2018;102(4):1903–1910.
[24] Schleifer KH, Kilpper-Bälz R. Transfer of *Streptococcus faecalis* and *Streptococcus faecium* to the genus Enterococcus nom. rev. as *Enterococcus faecalis* comb. nov. and *Enterococcus faecium* comb. nov. Int J Syst Evol Microbiol. 1984;34(1):31–34.
[25] Goldstein EJ, Tyrrell KL, Citron DM. *Lactobacillus* species: taxonomic complexity and controversial susceptibilities. Clin Infect Dis. 2015;60(Suppl. 2):S98–S107.
[26] Antony SJ, Stratton CW, Dummer JS. *Lactobacillus* bacteremia: description of the clinical course in adult patients without endocarditis. Clin Infect Dis. 1996;23(4):773–778.
[27] Murad CF, Sassone LM, Faveri M, et al. Microbial diversity in persistent root canal infections investigated by checkerboard DNA–DNA hybridization. J Endod. 2014;40(7):899–906.
[28] Tzanetakis GN, Azcarate-Peril MA, Zachaki S, et al. Comparison of bacterial community composition of primary and persistent endodontic infections using pyrosequencing. J Endod. 2015;41(8):1226–1233.
[29] Persoon IF, Buijs MJ, Özok AR, et al. The mycobiome of root canal infections is correlated to the bacteriome. Clin Oral Investig. 2017;21(5):1871–1881.
[30] Rôças IN, Jung IY, Lee CY, et al. Polymerase chain reaction identification of microorganisms in previously root-filled teeth in a South Korean population. J Endod. 2004;30(7):504–508.
[31] da Silva CC, Chaves Júnior SP, Pereira GL, et al. Antimicrobial photodynamic therapy associated with conventional endodontic treatment: a clinical and molecular microbiological study. Photochem Photobiol. 2018;94(2):351–356.
[32] Florio W, Cappellini S, Giordano C, et al. A new culture-based method for rapid identification of microorganisms in polymicrobial blood cultures by MALDI-TOF MS. BMC Microbiol. 2019;19(1):1–7.
[33] O'Connell LM, Santos R, Springer G, et al. Site-specific profiling of the dental mycobiome reveals strong taxonomic shifts during progression of early-childhood caries. Appl Environ Microbiol. 2020;86(7):1–16.
[34] Bowen WH. Dental caries – not just holes in teeth! A perspective. Mol Oral Microbiol. 2016;31(3):228–233.
[35] Kim AR, Ahn KB, Yun CH, et al. *Lactobacillus plantarum* lipoteichoic acid inhibits oral multispecies biofilm. J Endod. 2019;45(3):310–315.
[36] Twetman S. Are we ready for caries prevention through bacteriotherapy? Braz Oral Res. 2012;26(SPE1):64–70.
[37] Nadelman P, Magno MB, Masterson D, et al. Are dairy products containing probiotics beneficial for oral health? A systematic review and meta-analysis. Clin Oral Invest. 2018;22(8):2763–2785.
[38] Tahmourespour A, Kasra-Kermanshahi R, Salehi R. *Lactobacillus rhamnosus* biosurfactant inhibits biofilm formation and gene expression of caries-inducing *Streptococcus mutans*. Dent Res J. 2019;16(2):87–94.
[39] Schwendicke F, Dörfer C, Kneist S, et al. Cariogenic effects of probiotic *Lactobacillus rhamnosus* GG in a dental biofilm model. Caries Res. 2014;48(3):186–192.
[40] Dos Santos CS, Dos Santos LS, De Souza MC, et al. Non-opsonic phagocytosis of homologous non-toxigenic and toxigenic *Corynebacterium diphtheriae* strains by human U-937 macrophages. Microbiol Immunol. 2010;54(1):1–10.
[41] Cannon JP, Lee TA, Bolanos JT, et al. Pathogenic relevance of *Lactobacillus*: a retrospective review of over 200 cases. Eur J Clin Microbiol Infect Dis. 2005;24(1):31–40.
[42] Ikeda T, Yasui C, Hoshino K, et al. Influence of lactic acid bacteria on longevity of *Caenorhabditis elegans* and host defense against *Salmonella enterica* serovar Enteritidis. Appl Environ Microbiol. 2007;73(20):6404–6409.
[43] Fasseas MK, Fasseas C, Mountzouris KC, et al. Effects of *Lactobacillus salivarius*, *Lactobacillus reuteri*, and *Pediococcus acidilactici* on the nematode *Caenorhabditis elegans* include possible antitumor activity. Appl Microbiol Biotechnol. 2013;97(5):2109–2118.
[44] Lapirattanakul J, Nomura R, Okawa R, et al. Oral lactobacilli related to caries status of children with primary dentition. Caries Res. 2020;54(2):194–204.
[45] Klein MI, Flório FM, Pereira AC, et al. Longitudinal study of transmission, diversity, and stability of *Streptococcus mutans* and *Streptococcus sobrinus* genotypes in Brazilian nursery children. J Clin Microbiol. 2004;42(10):4620–4626.