JOURNAL OF ORAL MICROBIOLOGY
2025, VOL. 17, 2469894
https://doi.org/10.1080/20002297.2025.2469894

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RESEARCH ARTICLE

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In vitro modulation of proinflammatory and proteolytic activities of *Porphyromonas gingivalis* by selected lactobacilli

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ABSTRACT

Objective: The aim of the present study was to characterize the antimicrobial and anti-inflammatory activities of postbiotics from lactic acid bacteria against *Porphyromonas gingivalis*.

Material and methods: The anti-*P. gingivalis* activity of postbiotics from the CERELA culture collection was assessed by measuring changes in the expression of key host proteins by ELISA and qPCR, the proteolytic activity by a fluorescence and a spectrophotometric method and virulence factors from *P. gingivalis* by qPCR.

Results: Even though *Lacticaseibacillus (L.) rhamnosus* CRL1522 and *Lactiplantibacillus (L.) plantarum* CRL1363 exhibit only a discrete antibacterial activity against *P. gingivalis*, the cell-free supernatants of these strains significantly reduced *P. gingivalis*-induced secretion of interleukins IL-6 and IL-8 by keratinocytes and TNF-α and IL-6 by U937 macrophage-like cells. More importantly, *P. gingivalis* arginine-gingipain (Rgp) protease activity was markedly reduced by both lactic acid bacteria (LAB) strains. This finding is particularly interesting because it means that both LAB might prevent the ulterior citrullination of peptides and the consequent generation of autoantibodies. The expression of *COX2* and *TLR2* was also significantly downregulated in macrophages.

Conclusion: Postbiotics from *L. rhamnosus* CRL1522 and *L. plantarum* CRL1363 rise as suitable candidates for antagonizing the periodontopathogen *P. gingivalis*, since they were able to reduce the expression of proinflammatory cytokines and the protein degradation induced by this pathogen. We propose that postbiotics from these LAB could potentially halt the progression of periodontitis based on this *in vitro* study.

KEY MESSAGES

(1) *L. rhamnosus* CRL1522 and *L. plantarum* CRL1363cell-free supernatants reduced *P. gingivalis*-induced secretion ofinterleukins IL-6 and IL-8 by keratinocytes and TNF-α and IL-6 by U937macrophage-like cells.
(2) *L. rhamnosus* CRL1522and *L. plantarum* CRL1363 cell-freesupernatants significantly reduced the *P.gingivalis* protease activity and particularly the Rgp activity.
(3) Both CFS also reduce the expression of *COX2* and *TLR2* inmacrophages.

ARTICLE HISTORY
Received 7 November 2024
Revised 28 January 2025
Accepted 14 February 2025

KEYWORDS
Lactobacillales; proteolysis; cytokines; periodontitis; cell culture techniques

Introduction

Porphyromonas gingivalis is a Gram-negative anaerobic bacterium that plays a significant role in the development and progression of periodontitis [1]. This disease is caused by the presence of dysbiotic bacterial dental biofilm in the gingival sulcus, prompting immune cells to infiltrate the gingival sulcus. This process leads to inflammation, destruction of the supporting tissues of the teeth, bone resorption, and ultimately, tooth loss [2]. Dysbiotic dental biofilm shows increased abundance of periodontal pathogenic bacteria, among others *Porphyromonas gingivalis*, *Treponema denticola* and *Tannerella forsythia* [3]. It is important to stress that even though *P. gingivalis* is rather a quantitatively minor constituent, it is the key player promoting the emergence of dysbiosis and the progression of periodontitis [4].

It has been recently reported that *P. gingivalis* or its metabolites can potentially migrate to extra-oral sites and by implicated in site-specific diseases. For example, *P. gingivalis* among other periodontitis-related bacteria has been linked to gastrointestinal dysfunction. They

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Supplemental data for this article can be accessed online at https://doi.org/10.1080/20002297.2025.2469894

are able to reach the gut, inducing gut inflammation and a marked dysbiosis that can lead to or worsen a number of gut disorders such as Crohn's disease [5]. As a matter of fact, *P. gingivalis* can induce an increase in intestinal permeability, aggravating colitis and systemic inflammation in Crohn's disease patients [6]. Furthermore, it has been proposed that *P. gingivalis* can constitute a major risk factor for neurodegenerative diseases [7]. Indeed, the lipopolysaccharides (LPS) from gut bacteria have been shown to regulate the aggregation and toxicity of α-synuclein that in turn can propagate to the brain via the vagus nerve [8,9]. This model has been proposed to explain the onset of Parkinson's disease and it has been successfully tested already [10]. Alzheimer's disease has also been connected with dysbiosis and an initial infection has been proposed as one of the earliest events in the disease. Kumar et al. unexpectedly found that amyloid beta peptide (Aβ) was overproduced upon infection in the brain, suggesting that this peptide may play a protective role in innate immunity. It can be hypothesized that an aging blood-brain barrier would be leakier thus letting bacteria get to the brain, where the Aβ production would be triggered. These data suggest a dual protective/damaging role for Aβ, identifying inflammatory pathways as key factors in the development of the disease [11]. In addition, LPS from the microbiota can disrupt the integrity of the blood-brain barrier [12]. In this regard, *P. gingivalis* is one of the pathogens associated with Alzheimer's disease [13]. For all this, the control of gingivitis and periodontitis is of utmost importance.

Currently, periodontitis is managed through periodontal therapies, such as scaling and root planing, to remove dental plaque, which can be combined with clinical antibiotics in severe cases [14]. Yet, *P. gingivalis* can invade periodontal tissues minimizing the efficacy of mechanical debridement and resulting in chronic inflammation [15]. Moreover, conventional antibiotics have a negative impact on the microbiota and favors the emergence of bacterial resistance [16]. Therefore, alternative treatments are needed. For instance, proanthocyanidins proved to be useful for improving oxidative stress induced by *P. gingivalis* LPS [17]. Besides phenolic compounds, lactic acid bacteria (LAB) emerge as another plausible strategy. LAB strains or their metabolites have demonstrated antibacterial, antioxidant, and antifungal properties, as well as immunomodulatory capacities against oral pathogens [18,19]. Consequently, it can be proposed that LAB may help mitigate the proliferation of periodontopathogens or serve as an adjunct to periodontal treatments [20–22]. In turn, the control of periodontopathogens by LAB can be an invaluable tool for tackling neurodegenerative diseases such as Parkinson's and Alzheimer's [23].

In this regard, we have recently shown that *Lactobacillus delbrueckii* subsp. *lactis* CRL 581, a strain from the CERELA culture collection, is a promising candidate for the production of postbiotics for Alzheimer's disease therapy not only because of the antioxidant activity but also due to the inhibition of the acetylcholinesterase activity and the down-regulation of proinflammatory cytokines [24]. Postbiotics, i.e. preparation of inanimate microorganisms and/or their components that confers a health benefit on the host [25], may represent a better alternative to live bacteria, as they are more stable and have a longer shelf life. Moreover, the use of live bacteria poses risks such as alterations to the normal microbiota, tissue translocation, and even sepsis. Importantly, the potential spread of antibiotic resistance or virulence factors is completely avoided when choosing postbiotics over probiotics or living bacteria [26]. Based on these results, we sought LAB strains capable of antagonizing *P. gingivalis* in an *in vitro* setup, where *P. gingivalis* infection and postbiotics from LAB are administered simultaneously. We identified two strains from the CERELA culture collection that meet this criterion: *Lacticaseibacillus rhamnosus* CRL1522 and *Lactiplantibacillus plantarum* CRL1363, whose cell-free supernatants are promising candidates for combating *P. gingivalis* infections.

Materials and methods

Bacterial strains

Loigolactobacillus coryniformis CRL1001, *Limosilactobacillus reuteri* CRL1098, *Lactobacillus acidophilus* CRL43, *Enterococcus mundtii* CRL35, *Levilactobacillus brevis* CRL2013, *Lactobacillus delbrueckii* subsp. *lactis* CRL581 have been isolated from fermented food, whereas *Lacticaseibacillus rhamnosus* CRL1344, *Lacticaseibacillus rhamnosus* CRL1522, *L. acidophilus* ATCC4356, *Ligilactobacillus salivarius* sub *salivarius* ATCC 11741, *Lactiplantibacillus plantarum* CRL1363, and *Lacticaseibacillus rhamnosus* CRL1527 were isolated from human oral cavity. CRL strains listed belong to the CERELA Culture Collection. They were routinely grown in de Man Rogosa Sharpe (MRS) and Todd Hewitt (THB) (BD Bacto™ DIFCO®, Fisher Scientific) at 37°C under microaerophilic conditions.

Porphyromonas gingivalis (ATCC 33277) was grown in 5% blood agar according to CDC (MERK-Germany) or in Todd Hewitt broth (THB) in anaerobic conditions (90% N_2, 5% CO_2 and 5% H_2) at 37°C, supplemented with 10 mg/mL phytomenadione (Roche-Argentina) and 10 mg/mL hemin (Sigma). Mutant *P. gingivalis* KDP-112 (*rgpA*$^-$ and *rgpB*$^-$) (a kind gift from Professor Daniel Grenier, Oral Research Ecology Group, Université Laval, QC, Canada) was also cultured in enriched THB and cultivated in anaerobic conditions.

Overnight cultured cell free supernatants from the selected lactobacilli were harvested separately at 10,000 × *g* (SIGMA 3-18KS, Germany) and neutralized to pH 7 using NaOH 5 M with subsequent filtration (0.22 µm pore, Avantor VWR).

Eukaryotic cell cultures

Human gingival keratinocytes hTERT TIGKs (American Type Culture Collection (ATCC) CRL-3397, USA) [27] and the monocytic cell line U937 (ATCC CRL-1593.2) [28] were used as cell models. The keratinocytes were routinely grown in keratinocytes Serum Free Media (Life Technologies, USA) containing 100 µg/mL penicillin/streptomycin +0.25 µg/mL amphotericin B at 37°C in a humidified atmosphere of 5% CO_2. U937 monocytic cell line was cultured in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum (FBS) with 2 mm L-glutamine, 100 µg/mL penicillin/streptomycin +0.25 µg/mL amphotericin B. Differentiation to macrophage-like cells was induced by supplementation with 50 ng/mL phorbol 12-myristate 13-acetate (PMA, Sigma Aldrich) for 72 h.

Screening of anti-porphyromonas strains

We evaluated the proliferation of *P. gingivalis* in the presence of the different LAB strains. LAB were grown 16 h, centrifuged at 6,000 × g and resuspended in the same volume on Brain heart infusion broth (BHI) (Britania-Argentina) at final $OD_{600\,nm}$: 3. Colonies of *P. gingivalis* grown for 48 h were resuspended in BHI supplemented with 0.5 g/L L-cysteine, phytomenadione and hemin at final $OD_{600\,nm}$: 0.1. CDC-blood agar plates were spotted with 10 µL of each suspension, taking care of leaving each *P. gingivalis* spot close to the corresponding LAB sample. The same volume of BHI was used as a negative control and chlorhexidine digluconate (PLAC-OUT- Bernabó Labs) as positive control. Plates were incubated 48 h at 37°C in anaerobic conditions.

Antimicrobial activity displayed by L. rhamnosus CRL1522 and L. plantarum CRL1363 cell-free supernatants

P. gingivalis ATCC33277 growth was assessed following the optical density at 600 nm after 24 and 36 h incubation in enriched THB supplemented with CRL1522 cell-free supernatant (CFS) and CRL1363 CFS. The supernatants were neutralized first with 5 M NaOH and then filtered through a 0.22 µm sterile filter. The experiment was carried out in three independent experiments in polystyrene 96-well plate (Avantor VWR, North America) with a final volume of 100 µL.

Inhibition of the proteolytic activity

We tested the proteolytic activity displayed by *P. gingivalis* and the possible inhibition by *L. rhamnosus* CRL1522 and *L. plantarum* CRL1363 CFS. Suspensions of non-proliferative *P. gingivalis* cells (NPC) $OD_{600\,nm}$: 0.1 were preincubated for 10 minutes in a modified activity buffer: 10 mm L-cysteine, 0.4 M MOPs buffer, pH 7.5 containing 5 mm $CaCl_2$. *P. gingivalis* samples were incubated in the presence or absence of *L. rhamnosus* CRL1522 and *L. plantarum* CRL1363 CFS. Then α-casein was added as the substrate. After the incubation was completed, protein degradation was estimated by assessing the increase in primary amino groups upon α-casein proteolysis in smaller peptides. For this purpose, 1 mg/mL fluorescamine solution in acetone was added after α-casein incubation with *P. gingivalis* samples. Fluorescamine is a non-fluorescent molecule that becomes highly fluorescent upon reacting with primary amines, including amino acids and amino groups of peptides and proteins (λ_{exc} 390 nm, λ_{em} 470 nm). The samples were incubated at room temperature in the dark for 10 minutes, and the fluorescence was measured in an PC1 spectrofluorometer (ISS Inc., USA).

Gingipain activity and expression of virulence factors

P. gingivalis ATCC33277 and *P. gingivalis* KDP-112 were grown anaerobically for 48 h. $OD_{600\,nm}$ was adjusted to 0.4 and 50 µL of these suspensions were added to wells of a 96-well plate containing the chromogenic substrate *N*-α-benzoyl-DL-Arg-pNA (Sigma-Aldrich) for measuring the Rgp (arginine-gingipain) activity, as described previously by Aduse-Opoku et al. [29]. The absorbance at 405 nm was determined in an xMark™ microplate absorbance spectrophotometer (Bio-Rad).

P. gingivalis ATCC33277 was cultured in polystyrene 6-well plate (Avantor VWR, North America) for 24 h in enriched THB medium supplemented with CRL1522 and CRL1363 CFS. Total RNA isolation was performed with GeneJET RNA Purification Kit according to the manufacturer's instructions (ThermoFisher Scientific). Total RNA purity and quantity were analyzed by NanoDrop ONE (ThermoFisher Scientific). cDNA synthesis was carried out in a C1000™ Touch thermal cycler (Bio-Rad) using the High-Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific). WISENT ADVANCED qPCR master mix (Wisent, Quebec, Canada) was used for real time qPCR. The samples were denatured for 3 min at 95°C, then PCR reactions were cycled 40 times using the following parameters: 95°C for 20 s, 60°C for 20 s, and 72°C for

Table 1. Primers used for quantification of *P. gingivalis* gene expression by qPCR.

gene	primer forward	primer reverse
16S	5′-AGAGTTTGATCCTGGCTCAG-3′	5′-GGTTACCTTGTTACGACT-3′
fimA	5′-CAGCAGGAAGCCATCAAATC-3′	5′-CAGTCAGTTCAGTTGTCAAT-3′
mfa-1	5′-ATCTTCAGCACTCTCCACAAG-3′	5′-TTGTTGGGACTTGCTGCTCTTG-3′
rgp-A	5′-GGATGGTACTGCATCCGTTAAT-3′	5′-CTTCCACCACCTTCGCTTATAG-3′
kgp	5′-GCTGACAAAGGTGGAGACCAAAGG-3′	5′-TGTGGCATGAGTTTTTCGGAACCGT-3′

20s using a CFX Opus 384 Real time PCR system. (Bio-Rad). The primers used for assessing the expression of gingipain genes *rgp-A* (arginine-gingipain) and *kgp* (lysine-gingipain) are listed in Table 1. Since fimbriae proteins are also well-known *P. gingivalis* virulence factors, the expression of the long fimbriae FimA (*fimA*) and the short fimbriae Mfa1 (*mfa1*) was also assessed. 16S was used as a reference gene.

Cytokines production and expression of other inflammatory markers

Confluent hTERT TIGKs and U937 macrophage-like cell monolayers were washed twice with serum-free and antibiotic-free media and incubated one day with fresh medium without antibiotics prior to pretreatment with CRL1522 and CRL1363 CFS for 2 h. After that, cell cultures were stimulated with Heat-Inactivated (HI) *P. gingivalis* (multiplicity of infection of 10, i.e. 10 bacteria for 1 eukaryotic cell) for 24 h. *P. gingivalis* had to be added as heat-inactivated because of its strong proteolytic activity that interferes with the measurement of cytokines in the extracellular media [30]. Cells stimulated with the vehicle were used as the control. The supernatants were collected and stored at −30°C until use. Interleukin levels were measured by ELISA: IL-6 and IL-8 in hTERT TIGKs samples, whereas IL-6 and TNFα were assessed in U937 samples. For these measurements, both R&D Systems and Invitrogen kits were used.

The U937 cells were scraped, and then total RNA was extracted by means of the GeneJET RNA purification kit. The purity and quantity of total RNA were analyzed by NanoDrop ONE (ThermoFisher Scientific). cDNA synthesis and the quantification of the expression of the cyclooxygenase-2 gene (*COX2*) as well as the nucleotide-binding oligomerization domain-containing protein 1 (*NOD1*), nucleotide-binding oligomerization domain-containing protein 2 (*NOD2*), toll-like receptor 2 (*TLR2*) and toll-like receptor 4 (*TLR4*) genes was carried out as described above for bacterial expression of gingipains and fimbriae. Actin beta (*ACTB*), peptidylprolyl isomerase A (*PPIA*) and ribosomal protein lateral stalk subunit P0 (*RPLPO*) were used as reference genes. Primers used are listed in Table 2.

Statistical analyses

The statistical analyses were performed using GraphPad Prism (version 10.4.1, Boston, MA, USA). Normality was tested and then group comparisons were determined using one-way ANOVA with the Fisher's LSD *post hoc* test to assign the statistical significance ($p < 0.05$). Experiments were performed in 3 independent replicates and in two technical replicates for each condition.

Results

L. rhamnosus *CRL1522 and* L. plantarum *CRL1363 as anti-*P. gingivalis *strain*

We searched for LAB with antagonistic activity against the periodontopathogen *P. gingivalis*. For that purpose, twelve strains from the CERELA culture collection were tested. Most of them did not significantly affect the growth of *P. gingivalis* in solid media. However, results depicted in Table 3 shows that *L. rhamnosus* CRL1522 and *L. plantarum* CRL1363 did display an antagonistic activity against *P. gingivalis*. Representative spots from where Table 3 was constructed are shown in Supplementary figure S1. In fact, the growth spot of the periodontopathogen on agar medium was deformed due to the presence of these LAB strains. It is important to note that LAB spots contain both cells and culture medium.

Table 2. Primers used for quantification of mammalian gene expression by qPCR.

gene	primer forward	primer reverse
NOD1	5′-GTCACTGAGGCTCATCTGAAC-3′	5′-CATCCACTCCTGGAAGAACCT-3′
NOD2	5′-CATGTGCTGCTACGTGTTCTC-3′	5′-CCTGCCACAATTGAAGAGGTG-3′
COX2	5′-CTGGCGCTCAGCCATACAG-3′	5′-CGCACTTATACTGGTCAAATCCC-3′
TLR4	5′-TGGATACGTTTCCTTATAAG-3′	5′-GAAATGGAGGCACCCCTTC-3′
TLR2	5′-GCCAAAGTCTTGATTGATTGG-3′	5′-TTGAAGTTCTCCAGCTCCTG-3′
ACTB	5′-CCAACCGCGAGAAGATGA-3′	5′-CCAGAGGCGTACAGGGATAG-3′
PPIA	5′-CCACCAGATCATTCCTTCTGTAGC-3′	5′-CTGCAATCCAGCTAGGCATGG-3′
RPLPO	5′-CCTCGTGGAAGTGACATCGT-3′	5′-CTGTCTTCCCTGGGCATCAC-3′

Table 3. Screening of LAB with anti-*P. gingivalis* activity.

	LAB strain	activity in solid medium
1	*Loigolactobacillus coryniformis* CRL1001	-
2	*Limosilactobacillus reuteri* CRL1098	+
3	*Lactobacillus acidophilus* CRL43	-
4	*Enterococcus mundtii* CRL35	-
5	*Levilactobacillus brevis* CRL 2013	-
6	*Lactobacillus delbrueckii* subsp. *lactis* CRL581	+
7	*Lacticaseibacillus rhamnosus* CRL1344	+
8	*Lacticaseibacillus rhamnosus* CRL1522	++
9	*L. acidophilus* ATCC4356	+
10	*Ligilactobacillus salivarius* subsp *salivarius* ATCC 11741	-
11	*Lactiplantibacillus plantarum* CRL1363	++
12	*Lacticaseibacillus rhamnosus* CRL1527	+
13	cycloheximide	+++

Activity: +: low antagonistic effect, ++: intermediate antagonistic effect, +++: high antagonistic effect, -: no antagonistic effect.

We then narrowed down to two LAB strains and tested the anti-*P. gingivalis* activity of their cell-free supernatants. Figure 1 shows *P. gingivalis* growth after 24 and 36 h incubation in anaerobic conditions. Only *L. plantarum* CRL1363 CFS displayed a modest antibacterial activity against *P. gingivalis*.

L. rhamnosus CRL1522 and L. plantarum CRL1363 CFS reduce the Rgp proteolytic activity of P. gingivalis

Afterward, we focused on the possible antagonistic effect on the *P. gingivalis* proteolytic activity of the CFS from CRL1522 and CRL1563. For this purpose, we evaluated the inhibition of *P. gingivalis* proteases using a fluorescent probe that reacts with primary amino groups. α-casein was selected as the target protein because it has been demonstrated to be a suitable substrate for *P. gingivalis* proteases [31]. As it can be seen in Figure 2a, the fluorescence of α-casein samples is rather low but significantly increases upon addition of *P. gingivalis* extracts due to the activity of total *P. gingivalis* proteases. This activity was dramatically reduced when CRL1522 and CRL1563 CFS were present.

Since Rgp is the protease with more pathophysiological impact of *P. gingivalis* (see the Discussion section), we evaluated whether CRL1522 and CRL1563 CFS were able to reduce its activity. Both CFS reduced by 50% *P. gingivalis* Rgp activity as shown in Figure 2b. As expected, the negative control *P. gingivalis* KDP-112 displayed no Rgp activity.

Besides assessing the activity of Rgp, we have also quantified *P. gingivalis* gingipains and fimbriae gene expression following treatment with CRL1522 and CRL1563 CFS by RT-qPCR. As shown in Figure 3, there was no significant difference in the expression of *rgp* and *kgp* genes when CRL1522 and CRL1363 CFS were added, i.e. there was no downregulation of the protease gene expression due to the CFS. Therefore, the effect observed above (Figure 2b) was a direct effect on the activity, more likely as inhibitors

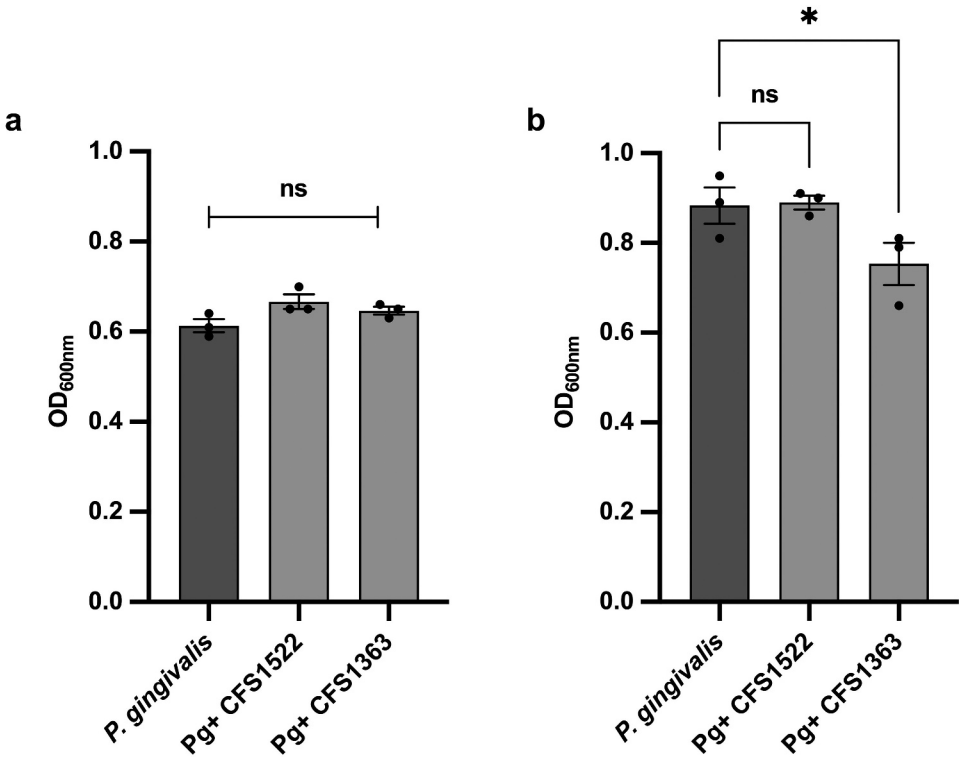

Figure 1. Antibacterial activity of CFS from CRL1522 and CRL1363. *P. gingivalis* (Pg) growth for 24 h (a) and *P. gingivalis* growth for 36 h (b) in anaerobic conditions in the presence or absence of neutralized CFS. *P. gingivalis* growth was estimated from the OD_{600nm}. All the data were expressed by means ±SEM. One-way ANOVA with a Fisher's LSD *post hoc* test. $N = 3$ independent experiments.

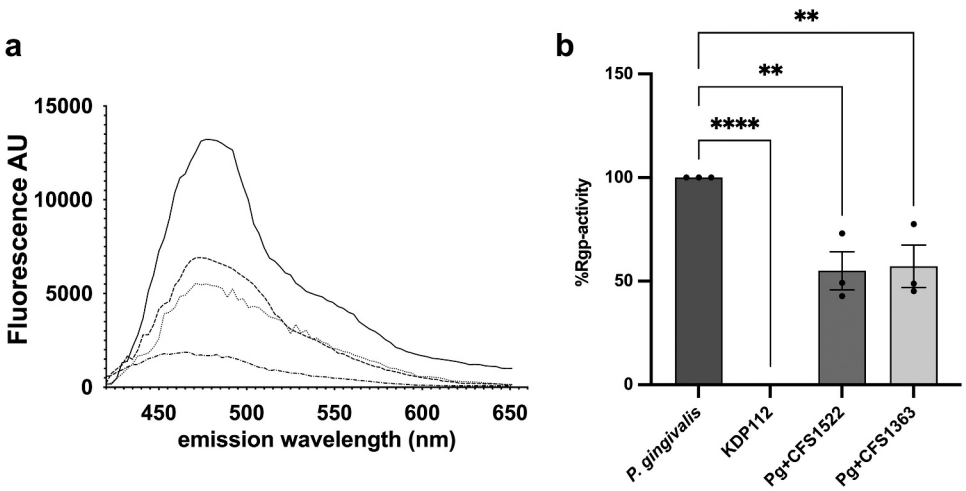

Figure 2.Inhibition of the *P. gingivalis* proteolytic activity. Total proteolytic activity assessed with α-casein as substrate (a). Samples were incubated with fluorescamine at room temperature in the dark as described in materials and methods and the emission fluorescence spectra were recorded from 420 nm to 650 nm (λexc 390 nm). α-casein (·–·), α-casein + gingipain-containing sample (—), α-casein + gingipain-containing sample + CFS from *L. rhamnosus* CRL1522 (···), α-casein + gingipain-containing sample + CFS from *L. plantarum* CRL 1363 (—). The spectra are representative of three independent experiments. Inhibition of Rgp (arginine-gingipain) activity (b). The percentage of gingipain activity inhibition in the samples containing CFS was calculated considering the hydrolysis of the chromogenic substrate by *P. gingivalis* ATCC33277 (Pg) as the 100% activity (0% inhibition). All the data were expressed by means ±SEM. One-way ANOVA with a Fisher's LSD *post hoc* test. *N*= 3 independent experiments.

of the protease activity. In addition, we verified that neither *P. gingivalis* fimbriae *fimA* and *mfa-1* gene expression was altered upon addition of the LAB CFS (Figure 3).

Proinflammatory cytokines secretion are reduced by CFS from CRL1522 and CRL1563

Given that gingival epithelial cells and monocytes/macrophages are key players regulating the inflammatory response in active periodontal lesion [32], we have investigated the anti-inflammatory potential of CRL1522 and CRL1363 CFS against *P. gingivalis*-induced inflammation by using *in vitro* cell culture models. First, we have quantified the expression of inflammation markers in U937 macrophage-like cells stimulated with heat-inactivated *P. gingivalis* by RT-qPCR. Even though we found no significant changes in the expression of either *NOD1*, *NOD2* and *TLR4* in U937 cells, we did find an increase in the expression of *TLR2* and *COX2* genes following stimulation with heat-inactivated *P. gingivalis*. Interestingly, there was a significant downregulation of these inflammation markers in the presence of CFS 1522 and CFS 1363, suggesting a modulation of the inflammatory response by the LAB supernatants (Figure 4).

Second, we measured the impact of CFS on the secretion of proinflammatory cytokines by U937 macrophage-like cells by ELISA. We observed that heat-inactivated *P. gingivalis* triggered an inflammatory response in U937 macrophages based on the levels of IL-6 and TNFα secreted in the presence of this pathogen (Figure 5). Importantly, the CFS from both LAB significantly reduced the secretion of IL-6, whereas the modulation of the TNFα concentration by the CFS did not show significant changes due to the dispersion of the data. However, a marked reduction of this cytokine upon addition of CRL1522 and CRL1563 CFS can be seen (Figure 5).

We also measured the anti-inflammatory potential of CRL1522 and CRL1563 CFS against heat-inactivated *P. gingivalis*-induced inflammation in hTERT TIGKs gingival keratinocytes. The addition of *P. gingivalis* triggered the keratinocytes inflammatory response by increasing IL-6 and IL-8 secretion (Figure 6). Preincubation of these cells with CRL1522 and CRL1563 CFS for 2 h prior stimulation with heat-inactivated *P. gingivalis* significantly reduced the secretion of IL-6 and IL-8 proinflammatory cytokines (Figure 6).

Discussion

In the present study we searched for LAB able to antagonize the periodontopathogen *P. gingivalis*. Two strains were selected, *L. rhamnosus* CRL1522 and *L. plantarum* CRL1363. Not surprisingly, both strains were isolated from the oral cavity, and therefore they shared the same environment with *P. gingivalis* which most likely made them more adapted to overcome the presence of this pathogen. The anti-*P. gingivalis* activities were characterized in cell culture experiments, where the challenge with this periodontopathogen was concomitant to the LAB postbiotics administration.

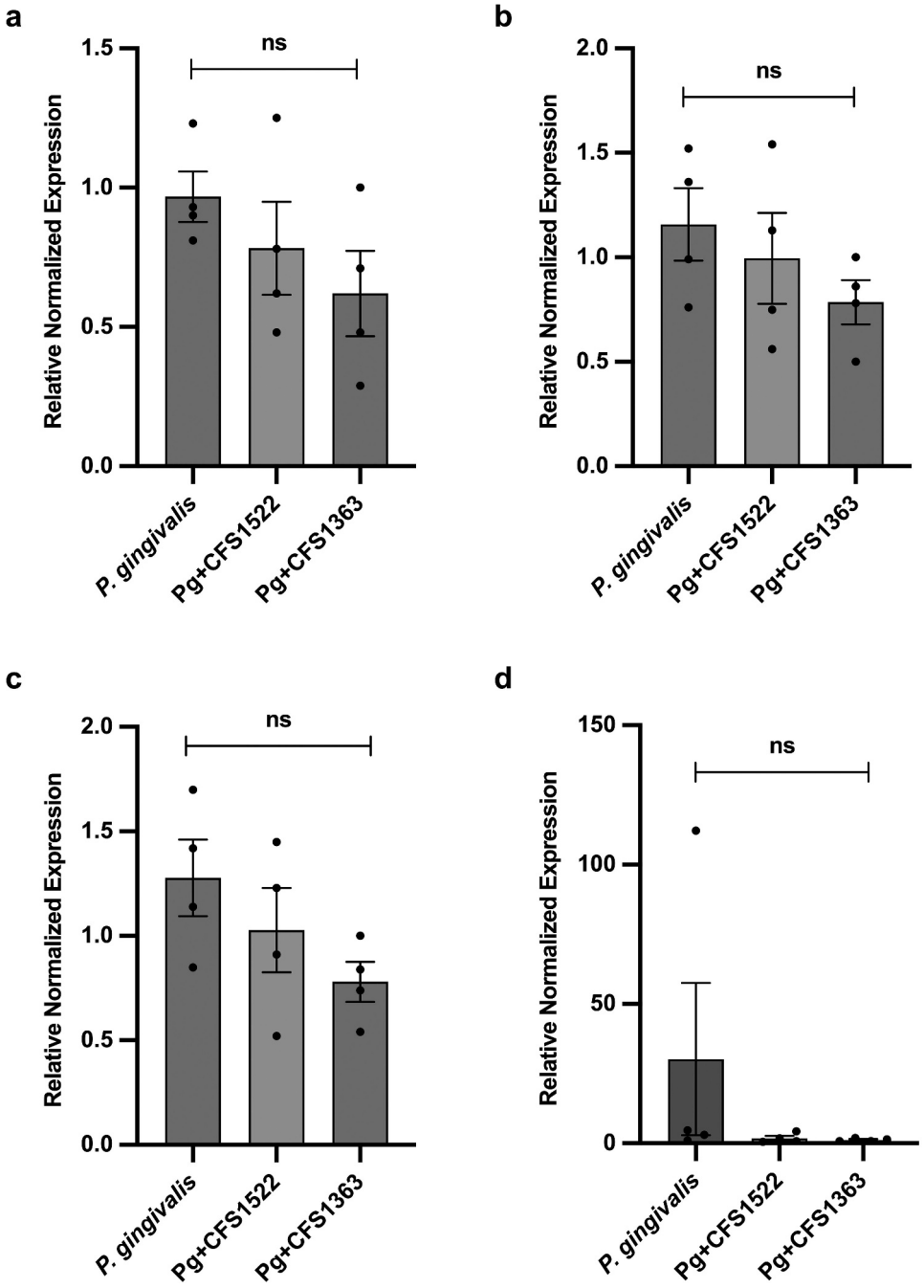

Figure 3.Gingipains and fimbriae gene expression by *P. gingivalis*. Relative normalized expression of *P. gingivalis* (Pg) *rgp* (a), *kgp* (b), *fimA* (c) and *mfa-1* (d). All the data were expressed by means ±SEM. One-way ANOVA with a Fisher's LSD *post hoc* test. $N = 3$ independent experiments.

Postbiotics from *L. rhamnosus* CRL1522 and *L. plantarum* CRL1363 were able to induce a significant reduction in the secretion of proinflammatory cytokines. In fact, the reduction of IL-6, IL-8 and TNFα secretion from macrophage-like cells and gingival keratinocytes is a very promising result. It has been shown that *P. gingivalis* induces the secretion of proinflammatory cytokines in epithelial cells-macrophages cocultures [33], in agreement with our results. These cytokines may contribute to the progression of periodontitis [34]. In the same trend, Charoensaensuk et al. showed that *P. gingivalis* infection increased intracellular reactive oxygen species production, activated NF-κB and the expression of IL-1β and TNF-α in mouse brain endothelial cells (bEnd.3; ATCC® CRL-2299 cells and primary mouse endothelial cells), which ultimately lead to apoptosis of endothelial cells [35]. *P. gingivalis*-induced inflammatory response was also observed in mouse models with an increased secretion of IL-6 and IL-8 cytokines [36].

In the present study, we found an upregulation of TLR2 but surprisingly not TLR4 in the presence of heat-inactivated *P. gingivalis* and a concomitant downregulation of TLR2 expression in the presence of both CFS. Even though both toll-like receptors are known to respond to LPS, experiments using TLR2/4 knockout macrophages, combined with TLR activation assays, revealed that TLR2 is the primary

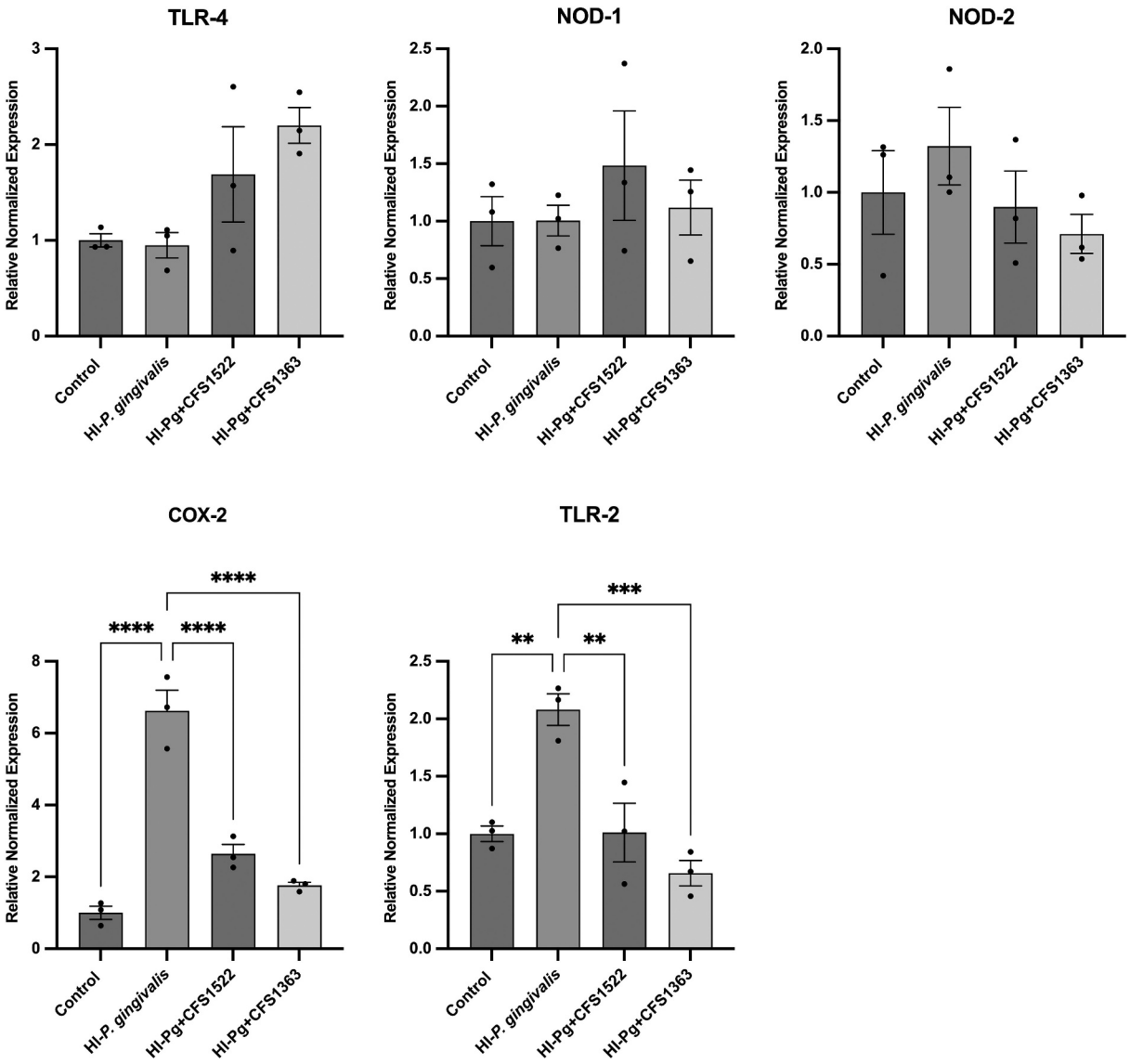

Figure 4. Expression of inflammatory markers in U937 cells. Relative normalized expression of *TLR4*, *NOD1*, *NOD2*, *COX2* and *TLR4* by RT-qPCR. Cells were treated with either 500 µL of CFS from *L. rhamnosus* CRL1522 or *L. plantarum* CRL1363 and stimulated or not with heat-inactivated (HI) *P. gingivalis* (Pg). 500 µL of THB was added to control. All the data were expressed by means ±SEM. The statistical analysis was performed with one-way ANOVA with Fisher's LSD *post hoc* test. $N = 3$ three independent experiments.

receptor responsible for activating responses to *P. gingivalis* LPS and whole cells [37]. Furthermore, *P. gingivalis* LPS can upregulate or downregulate TLR expression depending on the cell type, which includes the so-called TLR4 antagonism and the blockade of the TLR2 antimicrobial responses [4,38]. In this regard, Schön et al reported that a high concentration of LPS from *P. gingivalis* downregulates TLR4 [39]. Besides, NOD1 and NOD2 expression showed no significant differences in the presence of *P. gingivalis*, which is in agreement with the finding of Okugawa et al., who reported that *P. gingivalis* induces a weaker NOD1- and NOD2-stimulatory activities compared to other periodontopathogens, a feature important for its survival in the periodontal pocket [40].

Interestingly, the cyclic GMP-AMP synthase-stimulator of interferon genes (cGAS – STING) signaling pathway was identified as one of the mechanisms for the inflammatory response that leads to periodontitis in *P. gingivalis* infection [36]. Moreover, it has been proposed that systemic inflammation triggered by *P. gingivalis* may contribute to the development of Alzheimer's disease and the subsequent cognitive decline [41]. Importantly, it has been shown that local treatment of periodontitis decreases the serum levels of inflammatory factors [42]. However, whether local *P. gingivalis* infection treatment may contribute to relieve the progression or avoid the onset of Alzheimer's, Crohn's disease and other pathologies linked to periodontitis still needs experimental confirmation [43].

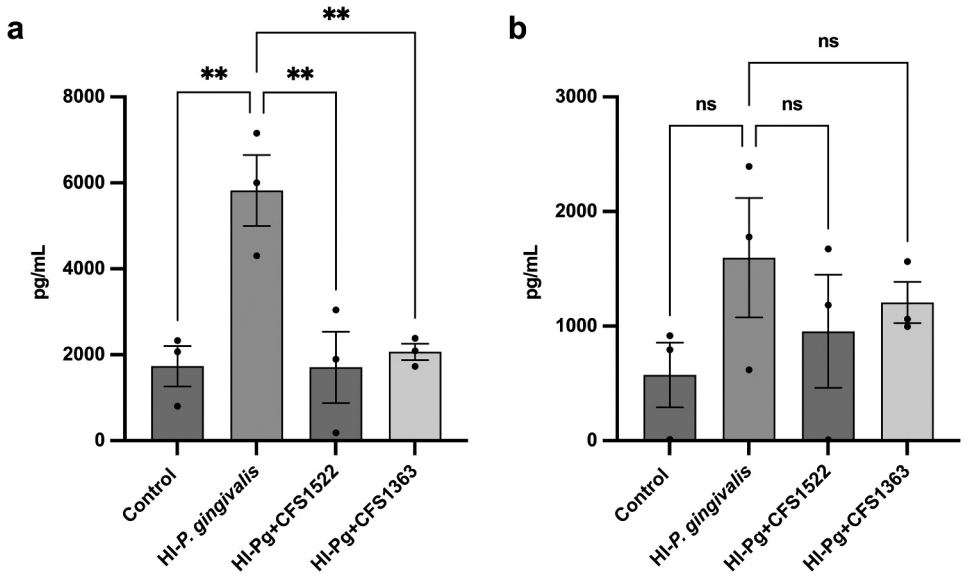

Figure 5.U937 macrophage-like cells proinflammatory cytokines secretion by ELISA. Cells were treated with either 500 µL of CFS from *L. rhamnosus* CRL1522 or *L. plantarum* CRL1363 and stimulated or not with Heat-Inactivated (HI) *P. gingivalis* (Pg). 500 µL of THB was added to control. IL-6 (A) and TNFα (B) were measured by ELISA in the cell culture supernatant. All the data were expressed by means ±SEM. One-way ANOVA with Fisher's LSD *post hoc* test. *N*= 3 three independent experiments.

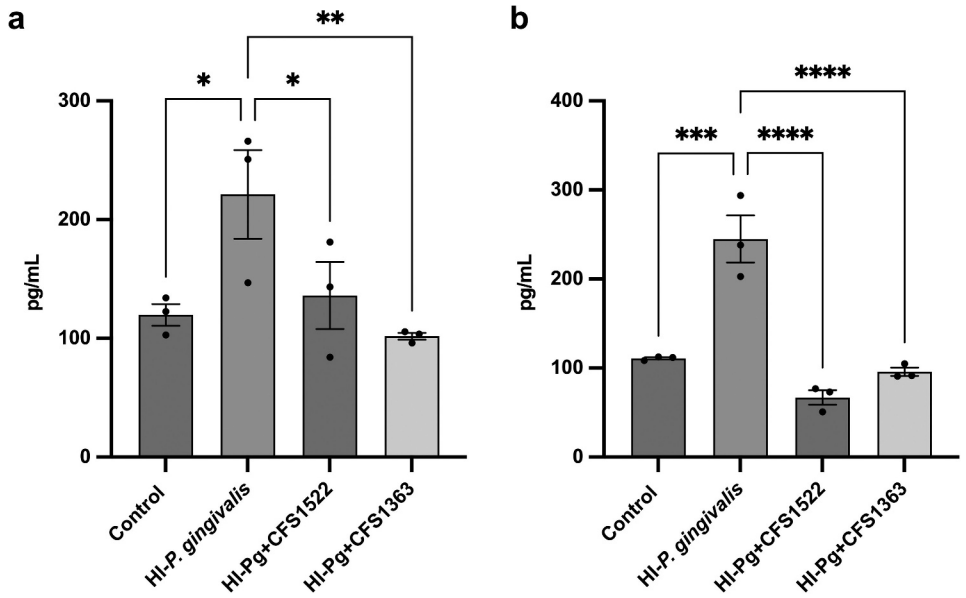

Figure 6.hTERT TIGKs cells proinflammatory cytokines secretion by ELISA. Cells were treated with either 500 µL of CFS from *L. rhamnosus* CRL1522 or *L. plantarum* CRL1363 and stimulated or not with Heat-Inactivated (HI) *P. gingivalis* (Pg). 500 µL of THB was added to control. IL-6 (a) and IL-8 (b) were measured by ELISA in the cell culture supernatant. All the data were expressed by means ±SEM. One-way ANOVA with Fisher's LSD *post hoc* test. *N* = 3 independent experiments.

Besides the increased cytokines secretion induced by *P. gingivalis* in our study, this pathogen also increased gingipain activity which was not associated with an increased gene expression. Gingipains are cysteine proteinases that are known as Rgp and Kgp because of their cleavage sites, i.e. Kgp cleaves the carboxy-terminal side of lysine residues in peptides, whereas Rgp cleaves the carboxy-terminal peptide bond of arginine residues [44]. Actually, Rgp can function as both an endopeptidase and an aminopeptidase [45]. Gingipains are crucial for obtaining nutrients from the environment and at the same time, they contribute greatly to the destruction of periodontal connective tissues and the development and maintenance of the inflammatory state in periodontal pockets [46]. Gingipains are thought to enhance the blood-brain barrier permeability thus contributing to development of neuroinflammation and the progression of neurodegenerative diseases [47]. *P. gingivalis* may also disrupt the intestinal barrier as it has been observed in the human colorectal adenocarcinoma-derived Caco-2 cell line [48]. It has been shown that *P. gingivalis* LPS alongside gingipains are key factors that contribute to the progression of the infection and

the inflammatory state associated with periodontitis [49]. Moreover, the inflammatory response induced by gingipains can be independent of their catalytic activity since heat-inactivated preparations are still effective [50]. Interestingly, the protease activity of gingipains also contributes to inflammation since they activate the protease-activated receptor (PAR) 2 in microglia, and a crosstalk between LPS and PAR2 was demonstrated [51].

In our study, we found that CFS of *L. rhamnosus* CRL1522 and *L. plantarum* CRL1363 can inhibit *P. gingivalis* protease activity, which actually includes not only gingipains but also collagenases and a dipeptidyl aminopeptidase IV [52]. Moreover, we found that the LAB supernatants significantly inhibited Rgp activity. This protease (both RgpA and RgpB) is particularly important because it can work alongside a peptidylarginine deiminase leading to the accumulation of citrullinated peptides [53]. In fact, Rgp cleaves fibrinogen and α-enolase, among other proteins, exposing C-terminal arginines that will undergo citrullination by the peptidylarginine deiminase [54]. This process leads to the generation of autoantibodies against citrullinated peptides, and an abnormal autoimmune response is raised [55]. Thus, Rgp emerges as an important target for combating *P. gingivalis* infection and a number of studies have focused on this protease both *in silico* and experimental approaches using natural and synthetic compounds [56–58].

In conclusion, postbiotics from *L. rhamnosus* CRL1522 and *L. plantarum* CRL1363 represent promising candidates for treating *P. gingivalis*-associated periodontitis since CFS can counteract the inflammatory response triggered by LPS and gingipains, including the proinflammatory activity of gingipains beyond their proteolytic activity. Therefore, our *in vitro* research, prior to animal and clinical studies, may provide a foundation for exploring critical questions in the fields of periodontal diseases and neurodegenerative disorders. For example, could targeting host tissue degradation and inflammation triggered by *P. gingivalis* using LAB postbiotics, rather than focusing solely on reducing periodontopathogen load, represent a potential strategy for combating neurodegenerative diseases such as Parkinson's and Alzheimer's?

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

Financial support was provided by Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT), PICT 2017 N° 0924, PICT 2019 N° 4126 and PICT 2021 N° 0354, Argentina to L.S and C.J.M. Financial support was provided by the Fondation de l'Université Laval (Fonds Émile-Beaulieu) to V.P.H. J.R.N.was the recipient of an ANPCyT doctoral fellowship. The Emerging Leaders in the Americas Program (ELAP) from Global Affairs Canada allowed J.R.N.to travel and work at Université Laval for an internship. E.M.H., M.E.F.N., L.S.and C.J.M. are career investigators of CONICET.

Author contribution statement:

Conceptualization, M.E.F.N., E.M.H., L.S., C.J.M. and V.P.H.; data acquisition: J.R.N and L.D., data analysis, E.M.H., L.S., C.J.M. and V.P.H.; funding acquisition, L.S. and V.P.H.; methodology, J.R.N., L.D., C.J. M., E.M.H. and V.P.H.; supervision, L.S., E.M.H, C.J. M. and V.P.H.; writing and editing, J.R.N., L.S., C.J.M. and V.P.H. All authors have approved the final manuscript.

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