Oral *Lactobacillus* strains reduce cytotoxicity and cytokine release from peripheral blood mononuclear cells exposed to *Aggregatibacter actinomycetemcomitans* subtypes in vitro

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**Abstract**

**Background:** This study evaluated the effect of oral lactobacilli on the cytotoxicity and cytokine release from peripheral blood mononuclear cells (PBMCs) when exposed to *Aggregatibacter actinomycetemcomitans* subtypes in vitro. The supernatants and cell wall extracts (CWEs) of eight *A. actinomycetemcomitans* strains, representing different subtypes, and three *Lactobacillus* strains were used. The PBMCs from six blood donors were exposed to supernatants and CWEs of *A. actinomycetemcomitans* or *Lactobacillus* strains alone or combinations and untreated cells as control. The cytotoxicity was determined by trypan blue exclusion method and IL-1β secretion by ELISA. TNF-α, IL-6, and IL-8 secretions were measured using Bioplex Multiplex Immunoassay.

**Results:** Supernatants or CWEs from all bacterial strains showed cytotoxicity and IL-1β secretion and the subtypes of *A. actinomycetemcomitans* showed generally a significantly higher effect on PBMCs than that of the *Lactobacillus* strains. Two highly toxic *A. actinomycetemcomitans* strains (JP2 and JP2-like) induced a higher response than all other strains. When combined, *Lactobacillus* significantly reduced the toxicity and the IL-1β secretion induced by *A. actinomycetemcomitans*. The effect varied between the subtypes and the reduction was highest for the JP2 and JP2-like strains. The *Lactobacillus paracasei* strain SD1 had a higher reducing effect than the other *Lactobacillus* strains. This strain had a consistent reducing effect on all subtypes of *A. actinomycetemcomitans* cytotoxicity, and release of IL-1β, IL-6, IL-8, and TNF-α from PBMCs of the blood donors. A strong and significant variation in cytokine release between the six blood donors was noticed.

**Conclusions:** *Lactobacillus* spp. and *L. paracasei* SD1 in particular, showed a limited but statistically significant reducing interaction with *A. actinomycetemcomitans* toxicity and release of cytokines in vitro.

**Keywords:** Microbial interaction, Oral lactobacilli, *Aggregatibacter actinomycetemcomitans*, Bacterial toxicity, Cytokine release, Peripheral blood mononuclear cells

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Background
Periodontitis is an inflammatory disease induced by the dental biofilm and affecting the tooth supporting tissues, bone and connective tissues, which may result in tooth loss. Several bacterial species have been associated with periodontitis and been designated major periodontopathogens [1]. Aggregatibacter actinomycetemcomitans has been associated with periodontitis in both young and older individuals [2, 3]. A. actinomycetemcomitans produces a leukotoxin (Lttx), an exotoxin targeting cells of the immune system in the periodontal tissues [4]. Leukotoxin induces a pro-inflammatory response by activation of macrophages/monocytes and secretions of IL-1β, and also selectively kills human leukocytes [5]. The leukotoxic activity among A. actinomycetemcomitans strains and subtypes is variable, and a highly toxic clone has been identified [6]. This genotype, termed the JP2 clone, primarily found in North and West African populations, has been closely associated with periodontitis in young individuals [6, 7]. Previous studies on various subtypes of A. actinomycetemcomitans strains isolated from an adult Thai population with periodontitis, revealed a significant variation in IL-8 cytokine expression (but not IL-1β, IL-6, and TNF-α) in human gingival epithelial cells (HGECS) [8, 9]. Non-serotypable strains (NS1 and NS2) and a JP2-like strain showed significantly lower IL-8 responses than the serotypable (serotype a-f) strains [9].

In the complex dental biofilm numerous interactions take place in order to regulate the microbial community by synergistic and antagonistic forces and metabolic networks [10]. Oral streptococci, which are abundant in most individuals, are considered to play a major role in regulating the dental biofilm ecology by producing interfering metabolic products and bacteriocins against a number of anaerobic gram-negative bacteria commonly associated with periodontitis including A. actinomycetemcomitans [11, 12]. Similarly, Lactobacillus species have shown inhibitory activities on the toxic effect of A. actinomycetemcomitans [13]. The purpose of the present study was to investigate the factors that are involved in the bacterial interaction between beneficial bacteria such as lactobacilli and a periodontopathogen such as A. actinomycetemcomitans. More specifically, the aim of this study was to examine the potential inhibiting or reducing effect of oral Lactobacillus strains on the cytotoxicity and secretion of the cytokines IL-1β, IL-6, IL-8 and TNF-α in peripheral blood mononuclear cells (PBMCs) by cell wall extracts (CWEs) and supernatants from a spectrum of A. actinomycetemcomitans subtypes.

Results
Cytotoxicity and IL-1β release from PBMCs by A. actinomycetemcomitans subtypes and Lactobacillus spp.
The cytotoxicity and IL-1β release upon stimulation with supernatant and CWEs of individual strains of lactobacilli and A. actinomycetemcomitans strains are shown in Fig. 1. Treatment of PBMCs with supernatants from the three lactobacilli strains resulted in lower cytotoxicity, approximately 1.2–2.5 folds (ranged 25.6 ± 4.8 to 28.7 ± 5.6%), and IL-1β secretion, approximately 1.5–3.3 folds (ranged 26.7 ± 5.2 to 36.9 ± 1.3 pg/mL), compared to when treated with A. actinomycetemcomitans strains NS1, NS2, JP2-like, and JP2 clone (p < 0.05, Fig. 1a and b). Also, NS2, JP2-like, and JP2 clone showed significantly higher cytotoxicity and IL-1β secretion compared to serotype a, serotype c, NS1, ATCC33384 (serotype c) and ATCC29523(serotype a) (p < 0.05, Fig. 1a and b). The significance for the higher release of IL-1β by the JP2 clone (OMG3952) in comparison with ATCC 33384 and ATCC 29523 was p < 0.041 and p < 0.026 respectively.

The percentage of cytotoxicity and IL-1β secretion induced by CWEs from A. actinomycetemcomitans strains was higher than lactobacilli strains, approximately 1.4–3.4 folds (ranged 50.4 ± 7.4 to 70.6 ± 2.1%) and 1.4–3.1 folds (ranged 31.7 ± 13.6 to 194.3 ± 45.4 pg/mL), respectively. Also, NS1, NS2, JP2-like, and JP2 clone showed significantly higher cytotoxicity and IL-1β secretion than serotype a, serotype c, ATCC33384 (serotype c) and ATCC29523 (serotype a) (p < 0.05, Fig. 1c and d).

The results of PBMCs treated with CWEs of tested strains were in line with the outcome of supernatant treatment by that A. actinomycetemcomitans strains and showed significantly greater cytotoxicity and IL-1β induction compared to lactobacilli strains (Fig. 1c and d).

The effect of Lactobacillus spp. on the cytotoxicity and IL-1β release from PBMCs by A. actinomycetemcomitans
The supernatant of Lactobacillus strains showed a varied reducing capacity on A. actinomycetemcomitans induced cytotoxicity and IL-1β release on PMBCs from six blood donors. L. paracasei SD1 reduced both cytotoxicity and IL-1β release from PBMCs by A. actinomycetemcomitans strains (mean value of 8 strains) significantly approximately 0.5–1.3 folds (from 58.9 ± 4.7 to 51.5 ± 3.7% and 0.7–1.2 folds (42.9 ± 4.6 to 29.1 ± 3.0 pg/mL; p < 0.05; Fig. 2a) and 0.5–1.2 fold reduction of IL-1β secretion (from 174.0 ± 30.8 to 143.1 ± 27.3 pg/mL, p < 0.001; Fig. 2b) of A. actinomycetemcomitans CWEs. Also, L. rhamnosus SD11 reduced significantly IL-1β secretion by A. actinomycetemcomitans CWEs 0.7–1.2 fold (from 174.0 ± 30.8 to 151.8 ± 28.9 pg/mL, p < 0.001; Fig. 2b). The cytotoxicity and IL-1β secretion did not reach statistical significance for A. actinomycetemcomitans CWEs combined with CWEs of LGG (0.6–0.9 fold reduction of cytotoxicity and 0.7–0.9 fold reduction of IL-1β secretion from
Effect of *Lactobacillus paracasei* SD1 on the cytotoxicity and IL-1β release by CWEs from *A. actinomycetemcomitans* subtypes

The cytotoxicity of individual *A. actinomycetemcomitans* strains after combination with *L. paracasei* SD1 was significantly lower compared with *A. actinomycetemcomitans* alone (*p* < 0.01; Fig. 4). The reducing effect of *L. paracasei* SD1 was 1.0–1.6 folds. NS1, NS2 and JP2-like strains revealed significant reduction compared to each of them alone showing 1.0–1.4 folds of reduction (Fig. 4). Individual variations on the response of the PBMCs were however, noticed.

The ability of *L. paracasei* SD1 to reduce cell responses induced by separate *A. actinomycetemcomitans* strains is shown in Fig. 3 and Fig. 4. The CWEs of *L. paracasei* SD1 proved to have a significant and consistent negative impact on IL-1β secretion in all cell cultures treated with the different *A. actinomycetemcomitans* strains (*p* < 0.01). The adding of *L. paracasei* SD1 to the cell cultures reduced the effect of each *A. actinomycetemcomitans* strain approximately 1.0–11.7 folds compared to when the cells were cultured with *A. actinomycetemcomitans* alone (Fig. 3). NS1, NS2, and JP2-like (ranged 128.1 to 261.0 pg/mL) showed a significant reduction approximately 1.2–10.2 folds compared with the addition of *L. paracasei* SD1 (ranged 25.6 to 174.3 pg/mL) in all subjects. Also, here, large individual variations were seen between the PBMCs.

**Effect of *A. actinomycetemcomitans* subtypes alone and combination with *L. paracasei* SD1 on IL-6, IL-8 and TNF-α release from PBMCs**

TNF-α (ranged 1256.6 to 51,103.8 pg/ml) showed the high levels when exposed to *A. actinomycetemcomitans* strains (Fig. 5) while IL-6 (ranged 10.34 to 631.9 pg/ml) and IL-8 (ranged 1009.9 to 15,480.6 pg/mL) were considerably lower than TNF-α (Fig. 6 and Fig. 7). After combination, *L. paracasei* SD1 reduced the secretion of IL-6, IL-8, and TNF-α significantly in all cell cultures also treated with the different *A. actinomycetemcomitans* strains (*p* < 0.05; Figs. 5, 6, 7). The reduced secretion of IL-6, IL-8, and TNF-α was 1.1–20.7, 1.1–3.4, and 1.1–
Fig. 2 The cytotoxicity (a) and IL-1β concentration (b) secreted from PBMCs (from six blood donors) exposed to the supernatant (1:1 concentration) or CWEs (100 μg/mL), where each spot represents one blood donor and a mean value of eight A. actinomycetemcomitans strains alone or in combination with three strains of Lactobacillus spp. (SD1, SD11 or LGG). * Statistically significant lower cytotoxicity (p < 0.001) or IL-1β concentration (p < 0.048) for the combinations compared to A. actinomycetemcomitans alone using Mann Whitney U test. The median of the group is shown as a horizontal line.
11.5 folds, respectively. Strong individual variations were also seen here.

Discussion
This study examined the effect of oral lactobacilli on the cytotoxicity and pro-inflammatory cytokine responses of PBMCs induced by eight different subtypes of *A. actinomycetemcomitans*. The main finding was that statistically significant reductions of the cytotoxicity and the pro-inflammatory cytokines, IL-1β, IL-6, IL-8, and TNF-α, was demonstrated for oral *Lactobacillus* strains, and *L. paracasei* SD1 in particular, when combined with the *A. actinomycetemcomitans* subtypes although a significant variation between the blood donors was noticed. The study illustrates the high complexity in the interaction between oral bacteria and the host that exists even in vitro between bacterial species, species subtypes and components and between host (blood donor) cells such as PBMCs.

All *A. actinomycetemcomitans* and *Lactobacillus* strains showed a toxic effect and induced the release of cytokines from PBMCs in various degrees, although *A. actinomycetemcomitans* showed a generally and statistically significant higher toxic effect than the *Lactobacillus* strains. This finding was for both CWEs and supernatant indicating that various cell wall components
as well as extracellular products from all bacteria, gram-negative as well as gram-positive, interfere with the host cells, in this case PBMCs. This means that the cytotoxicity as was measured in this study is of low specificity and basically not related to any particular component of the bacteria. This should be kept in mind for the complex in vivo situation with a dental biofilm with hundreds of different species in highly variable amounts. Thus, the effect of specific bacterial species, such as *A. actinomycetemcomitans* or lactobacilli, is generally unpredictable.

Not surprisingly the highly, according to the literature [6], toxic genotype JP2 of *A. actinomycetemcomitans* showed the highest toxic effect and significantly higher than the other *A. actinomycetemcomitans* subtypes. Similarly, the effect on IL-1β release was more pronounced for the JP2 clone, indicating that the effect on host cells may be specifically associated with leukotoxin production and with a potential co-stimulation of PBMCs by lipopolysaccharides (LPS). *A. actinomycetemcomitans* is a bacterial species associated with periodontitis due to its leukotoxin production, internalization into fibroblast cells, and immunomodulation [8, 9]. In a previous study it was shown that *A. actinomycetemcomitans* suppressed IL-8 mRNA expression and its function [9] as well as, this study revealed the potency of *A. actinomycetemcomitans* on stimulating PBMCs to secret IL-1β. 

![Fig. 4 Cytotoxicity of PBMCs from six blood donors exposed to CWEs (100 μg/mL) of each strain of *A. actinomycetemcomitans* and their combination with *Lactobacillus paracasei* SD1. *Statistically significant cytotoxicity for the combinations compared to *A. actinomycetemcomitans* alone (Wilcoxon Signed-Rank Test; \( p < 0.05 \)).](image)
inflammatory cytokines and chemokine, which is in accordance with the results of this study. *A. actinomy- ceteremcomitans* CWEs and supernatant showed significantly higher cytotoxicity and IL-1β secretion by PBMCs compared to lactobacilli. The response to the highly toxic JP2 genotype (OMGS 3952) indicates that the leukotoxin plays a major role in the IL-1β release from PBMCs.

*A. actinomycteremcomitans* components other than the toxin appeared to be stronger regulators of pro-inflammatory cytokines such as IL-6, IL-8, TNF-α in the various experiments. The release of the cytokines IL-6 and IL-8 was low to moderate (Fig. 6 and Fig. 7) while the release of TNF-α was remarkably high for all *A. actinomycteremcomitans* subgroups (Fig. 5). The JP2 subtype (strain OMG3952) showed here the lowest response except for one of the blood donors (Fig. 5, OMGS 3952), indicating that other bacterial components than leukotoxin may be responsible for the TNF-α release.

Both supernatants and CWEs of oral lactobacilli reduced the cytotoxicity and pro-inflammatory cytokines of the PBMCs when combined with an adjusted amount of supernatant and CWEs of *A. actinomycteremcomitans* strains. In particular, the supernatant and CWEs of *L. paracasei* SD1 had a significant and consistent reduction on IL-1β, IL-6, IL-8, and TNF-α release on all subtypes of *A. actinomycteremcomitans*. The component responsible for the reducing effect in lactobacilli is not known.

**Fig. 5** TNF-α secreted from PBMCs from six blood donors and exposed to CWEs (100 μg/mL) of each strain of *A. actinomycteremcomitans* and their combination with *Lactobacillus paracasei* SD1. * Statistically significant cytotoxicity for the combinations compared to *A. actinomycteremcomitans* alone (Wilcoxon Signed-Rank Test; *p* < 0.05)
Lactobacillus strains have been recognized as beneficial bacteria along the gastrointestinal tract and suggested and used as a probiotic due to its both general inhibiting effect on other microorganisms e.g. by its acid production [10, 11] and specific effect on bacterial growth [15] and reducing effect on toxicity by bacteriocins [13]. The potential interactions in the regulation of the dental biofilm ecology are numerous and involve many different microbial species and the importance of specific interaction between lactobacilli and A. actinomycetemcomitans, as was shown in this in vitro study, can only at this stage be speculated. The reducing effect of the oral Lactobacillus strains on the toxicity of A. actinomycetemcomitans could be considered limited from this in vitro study in view of the rare colonization of lactobacilli in the periodontal pocket, however it is possible that certain strains may interact with the colonization and growth of A. actinomycetemcomitans in the early stages of periodontal disease.

The response was highly variable between the blood donors for both IL-1β and TNF-α and even non-responders were noticed as illustrated by subject 6 (Fig. 3) in whom IL-1β was not detected for any of the 8 subtypes of A. actinomycetemcomitans. This variation in host susceptibility for a putative periodontal pathogen such as A. actinomycetemcomitans and cell components and extracellular products from them illustrates the complexity of the interaction between the dental biofilm and

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**Fig. 6** IL-6 secreted from PBMCs from six blood donors and exposed to CWEs (100 μg/mL) of eight strains of A. actinomycetemcomitans alone and in combination with Lactobacillus paracasei SD1. * Statistically significant cytotoxicity for the combinations compared to A. actinomycetemcomitans alone (Wilcoxon Signed-Rank Test; p < 0.05)
host tissues in vivo and the variation in the periodontal disease susceptibility. It could be argued that the variation seen between the blood donors may be due to methodological errors in collecting, storage and preparing the buffy coats for the experiments. All buffy coats were ordered and prepared from blood samples the day before the experiment. At the day of the experiment, the cells from various buffy coats were treated in parallel and in same and standardized way. We therefore argue that methodological errors have only a minor impact in this study. Instead, this study show that substantial and inter-individual variation exists in cytokine release from the monocytes. Thus, there is a benefit of using buffy coats in studies to include this natural variation among different subjects, which is not possible when using cell lines.

Conclusions
This study found a limited but statistically significant reducing effect of three oral Lactobacillus strains on the toxicity of eight Aggregatibacter actinomycetemcomitans strains. This in vitro study further demonstrates the complexity of the interactions among bacteria and also between bacteria and the host cells. The large variability among the target cells (A. actinomycetemcomitans), among the effector strains (lactobacilli), and among host cells (PBMCs from blood donors), illustrate this complexity. The most inhibited strains were the most toxic.

\[\text{IL-8 secreted from PBMCs from six blood donors and exposed to CWEs (100 \mu g/mL) of eight strains of A. actinomycetemcomitans alone and in combination with Lactobacillus paracasei SD1. * Statistically significant cytotoxicity for the combinations compared to A. actinomycetemcomitans alone (Wilcoxon Signed-Rank Test; } p < 0.05)\]

\[\text{Fig. 7}\]

*The figure shows the IL-8 secretion from PBMCs of six blood donors exposed to CWEs (100 \mu g/mL) of A. actinomycetemcomitans strains alone and in combination with Lactobacillus paracasei SD1. The bars indicate statistically significant cytotoxicity for the combinations compared to A. actinomycetemcomitans alone (Wilcoxon Signed-Rank Test; } p < 0.05).
target strains (the JP2 clone), and \textit{L. paracasei} SD1 had a more inhibiting effect than the other oral \textit{Lactobacillus} strains.

**Methods**

**Study design**

The following factors were tested:

- The toxicity and cytokine release for the individual strains of \textit{A. actinomyctemcomitans} and \textit{Lactobacillus} spp.
- The toxicity and cytokine release of supernatant and cell wall extract (CWEs) of each strain respectively.
- The toxicity and cytokine release of a strain of \textit{A. actinomyctemcomitans} alone and in combination with \textit{Lactobacillus} strains.
- The diversity of cytokine release between blood donors.

**Bacterial strains**

Five strains of \textit{A. actinomyctemcomitans} were selected among clinical isolates from Thai adults with periodontitis and representing different subtypes (serotype a and c, a JP2 like serotype c, and 2 non-serotypable strains, NS1 and NS2, representing two different DGGE subtypes that have shown a high toxicity in a previous study [8]) were included as target strains for the study together with 3 reference strains representing serotype a (ATCC29523), serotype c (ATCC33384), and the JP2 clone serotype b (OMGS 3952). The latter strain was isolated from a young girl of Cap Verde Island with an advanced periodontitis [16]. Further, \textit{Lactobacillus paracasei} SD1 and \textit{L. rhamnosus} SD11 previously tested as oral probiotic strains [17, 18] and a reference strain \textit{L. rhamnosus} ATCC53103 (LGG) were included as effector strains. The \textit{A. actinomyctemcomitans} strains were cultured in brain heart infusion broth (BHI, Acumedia, Lansing, Mich, USA) and were incubated at 37 °C for 48 h under anaerobic conditions.

**Bacterial supernatant preparation**

The concentrations of \textit{A. actinomyctemcomitans} were adjusted by the optical dentistry at OD$_{600}$ = 0.15 which corresponded to 10$^8$ CFU/mL in BHI broth and were incubated at 37 °C for 48 h under anaerobic conditions. Supernatants were collected after centrifugation at 2800 g for 10 min and then, the pH was adjusted to pH 7.0. The supernatants were filtered and stored at −20 °C until further analysis. The cell pellets were kept for cell wall extraction (see below).

The supernatant of \textit{Lactobacillus} strains was prepared in the same way as for \textit{A. actinomyctemcomitans}, although the concentration of \textit{Lactobacillus} cells was adjusted at OD$_{600}$ = 0.20) corresponding to cell counts of 10$^8$ CFU/mL.

**Bacterial cell wall preparation**

Cell pellets were used to extract cell wall components by differential centrifugation, as previously described [9]. Briefly, bacterial cells were resuspended in PBS pH 7.0 in the presence of a proteinase inhibitor cocktail (1 tablet yields a 1 mM EDTA solution in 10 ml, Roche Molecular Biochemicals, Mannheim, Germany) and the cells were disrupted by sonication. Intact cells were removed by centrifugation at 2200 g for 10 min at 4 °C, whereas the cell wall extract (CWEs) were collected from the supernatant by centrifugation at 30,000 g for 20 min at 4 °C. The cell wall pellet was resuspended in 500 μL of PBS with pH 7.0, and the total protein concentration was determined by the Bradford assay [19].

**Human peripheral blood mononuclear cells (PBMCs) isolation**

 Buffy coats were prepared from fresh blood collected from six healthy blood donors at hospital blood bank of the Sahlgrenska University Hospital in Gothenburg, Sweden and used for the experiments within 24 h. Theuffy coats were used after deidentification, and according to Swedish legislation section code 4 3p SFS 2003: 460, no informed consent is needed. The PBMCs isolated from theuffy coat from each donor were used for each co-incubation with all tested bacteria to analyze cytotoxicity and cytokine secretions. The experiments were run in triplicates for eachuffy coat.

Peripheral blood mononuclear cells (PBMCs) were isolated fromuffy coats by centrifugation over Ficoll-Paque™ Plus density gradient (GE healthcare Biosciences AB, Uppsala, Sweden). In brief, eachuffy coat was diluted 1:1 with PBS pH 7.0 and layered on 3 ml of Ficoll-Paque and centrifuged at 400 g for 30 min at room temperature. The PBMCs were collected at the interphase and washed twice in PBS and finally resuspended in Dulbecco’s Modified Eagle Medium plus GlutaMAX™ (Gibco, Life Technologies, Paisley, UK) supplemented with 5% human serum (Sigma-Aldrich, St Louis, MO, USA) and 1% penicillin-streptomycin (Sigma-Aldrich), then the cells were counted using a hemacytometer.

**Bacterial exposure**

The PBMCs were added to 96-well plates at 2 × 10$^6$ cells/well and cultured in the presence of the various concentrations (50, 100, 200, 400, 800, 1000 μg/mL) of lactobacilli CWEs and tested for cytotoxicity and cytokine secretion in order to find a suitable concentration for the experiments. A concentration of 100 μg/mL of lactobacilli CWEs was used while the concentration of \textit{A. actinomyctemcomitans} CWEs was obtained from our
previous study [9]. For the supernatant of both strains, an undiluted supernatant was used throughout this study.

PBMCs were added to 96-well plates at 2 × 10^6 cells/well and cultured in the presence of bacterial supernatants or 100 μg/mL CWEs of either A. actinomycetemcomitans or Lactobacillus strains alone or combinations for 2 h at 37 °C in 5% CO₂ incubator. PBMCs cultured without bacterial components were used as controls. The cell-free culture supernatants were collected for IL-1β, IL-6, IL-8, and TNF-α determination and the PBMC cells were collected for the cytotoxicity test.

**Cytotoxicity and IL-1β determination**

The cytotoxicity of A. actinomycetemcomitans or Lactobacillus strains, single or combinations, was determined using the modified trypan blue exclusion method [20]. The percentage of cytotoxicity was calculated by 100 – (surviving cells of the test/surviving cells of the control × 100).

IL-1β analysis of cell-free culture medium was performed using the DuoSet ELISA Development Kit (R&D Systems, Abingdon, UK) according to the manufacturer's instructions. The plates were coated with capture antibody overnight. The cell culture supernatants were incubated with a cytokine-specific biotinylated detection antibody and marked with streptavidin-conjugated horseradish-peroxidase. After the addition of the substrate, the absorbance was measured using an ELISA microplate reader (Synergy 2, BioTek Instruments, Inc., Winooski, VT, USA) at 405 nm and compared to a standard curve in order to calculate the concentration presented as pg/mL.

**IL-6, IL-8, and TNF-α determination**

The cytokines, IL-6, IL-8, and TNF-α, were measured using a custom-made multiplex assay (Bio-Plex Express Assay, Bio-Rad Laboratories, Hemel Hempstead, UK) based on Luminex xMAP technology according to the manufacturer’s instructions. Briefly, the standard was reconstituted and diluted in a fourfold dilution series. Antibody coupled capture beads were prepared and plated. After washing using a Bio-Plex Pro™ wash station (Biorad), diluted samples and standards were added to the beads in the wells. The plate was incubated on a shaker and after incubation and wash, detection antibodies were added to each well and after the streptavidin-phycocerythin solution (R&D Systems, Abingdon, UK) was added to the wells. In the last incubation step, beads were resuspended in assay buffer and the plate was read with a BioPlex 200 instrument equipped with BioManager analysis software (BioRad). The absolute concentrations of the samples were determined by comparing the bead colour and mean fluorescence intensity from each set of beads against an automatically optimized and manually verified standard curve. The cytokine concentration was presented as pg/mL.

**Statistical analysis**

The results of bacterial CWEs and supernatant on PBMCs stimulation were compared using Mann–Whitney U Test while the results of SD1 combined with A. actinomycetemcomitans on PBMCs stimulation were analyzed with Wilcoxon Signed-Rank Test. A p-value < 0.05 was considered as statistically significant.

**Abbreviations**

BHI: Brain heart infusion; CWE: Cell wall extract; DGGE: Denaturated gradient gel electrophoresis; HGEC: Human gingival epithelial cells; IL: Interleukin; LPS: Lipopolysaccharide; NS: Non-serotypable; PBMCs: Peripheral blood mononuclear cells; TNF-α: Tumor necrotic factor-alfa.

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**Authors’ contributions**

NP, RT and GD designed the study. NP performed the experiments and drafted the manuscript. NP, AB, AKO, RT and GD discussed the study design and data analysis and revised the manuscript. All authors read and approved the final manuscript.

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**Availability of data and materials**

All data generated or analyzed during this study are included in this published article.

**Ethics approval and consent to participate**

Ethics approval not applicable. Buffy coats were obtained from the hospital blood bank at Sahlgrenska University Hospital, Gothenburg, Sweden after deidentification, and according to the Swedish legislation section code 45 3p SFS 2003:460, no informed consent is needed. Permission to use blood-components for other purposes than transfusion was approved (171017) with the reference number (KTM) K 22/17.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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References

1. Socransky SS, Haffajee AD, Cupini MA, Smith C, Kent RL Jr. Microbial complexes in subgingival plaque. J Clin Periodontol. 1998;25:134–44.

2. Minguez M, Pouza X, Herrera D, Blasi A, Sanchez MC, Leon R, Sanz M. Characterization and serotype distribution of Aggregatibacter actinomycetemcomitans isolated from a population of periodontitis patients in Spain. Arch Oral Biol. 2014;59:1359–67.

3. Mombell A, Casagri F, Madrazo P. Can presence or absence of periodontal pathogens distinguish between subjects with chronic and aggressive periodontitis? A systematic review. J Clin Periodontol. 2002;29:105–21.

4. Johansson A. Aggregatibacter actinomycetemcomitans: Leukotoxin: a powerful tool with capacity to cause imbalance in the host inflammatory response. Toxins. 2011;3:242–59.

5. Kelk P, Abdul H, Claesson R, Sandstrom G, Sjostedt A, Johansson A. Cellular and molecular response of human macrophages exposed to Aggregatibacter actinomycetemcomitans leukotoxin. Cell Death Dis. 2011;2:1–10.

6. Haubek D. The highly leukotoxic JP2 clone of Aggregatibacter actinomycetemcomitans: evolutionary aspects, epidemiology and etiological role in aggressive periodontitis. APWIS. 2010;1:181–53.

7. Åberg CH, Haubek D, Kwamin F, Johansson A, Claesson R. Leukotoxic activity of Aggregatibacter actinomycetemcomitans and periodontal attachment loss. PLoS One. 2014;9:1–11.

8. Pahumunto N, Ruangprin P, Wongsuwanlert M, Piwat S, Dahlen G, Teanpaisan R. Aggregatibacter actinomycetemcomitans serotypes and DGGE subtypes in Thai adult chronic periodontitis. Arch Oral Biol. 2015;60:1789–96.

9. Pahumunto N, Chotjumlong P, Makeudom A, Krisanaprakornkit S, Dahlen G, Teanpaisan R. Pro-inflammatory cytokine responses in human gingival epithelial cells after stimulation with cell wall extract of Aggregatibacter actinomycetemcomitans subtypes. Anaerobe. 2017;48:103–9.

10. Huang R, Li M, Gregory RL. Bacterial interactions in dental biofilm. Virulence. 2011;5:435–44.

11. Standar K, Kreikemeyer B, Redans Z, Munter WL, Laue M, Podbielski A. Setup of an in vitro test system for basic studies on biofilm behavior of mixed species cultures with dental and periodontal pathogens. PLoS One. 2010;5:1–14.

12. Duan D, Scoffield JA, Zhou X, Wu H. Fine-tuned production of hydrogen peroxide promotes biofilm formation of Streptococcus parasanguinis by a pathogenic cohabitant Aggregatibacter actinomycetemcomitans. Environ Microbiol. 2016;18:4023–36.

13. Nissen L, Sgorbati B, Biavati B, Belibasakis GN. Lactobacillus salivarius and L. gasseri down-regulate Aggregatibacter actinomycetemcomitans exotoxins expression. Ann Microbiol. 2014;64:61–7.

14. Kelk P, Claessen R, Chen C, Sjostedt A, Johansson A. IL-1beta secretion induced by Aggregatibacter (Actinobacillus) actinomycetemcomitans is mainly caused by the leukotoxin. Int J Med Microbiol. 2008;298:529–41.

15. Jones SE, Versalovic J. Probiotic Lactobacillus reuteri biofilms produce antimicrobial and anti-inflammatory factors. BMC Microbiol. 2009;9:1–9.

16. Haubek D, Poulsen K, Westergaard J, Dahlen G, Killan M. Highly toxic clone of Actinobacillus actinomycetemcomitans in geographically widespread cases of juvenile periodontitis in adolescents of African origin. J Clin Microbiol. 1996;34:1576–8.

17. Teanpaisan R, Piwat S. Lactobacillus paracasei SD1, a novel probiotic, reduces mutans streptococci in human volunteers: a randomized placebo-controlled trial. Clin Oral Invest. 2014;18:857–62.

18. Teanpaisan R, Piwat P, Tianwisit S, Sophatha B, Kampoo T. Effect of long-term consumption of Lactobacillus paracasei SD1 on reducing gut streptococci and caries risk: a randomized placebo-controlled trial. Dent J. 2015;3:43–54.

19. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1979;72:248–54.

20. Pahumunto N, Ruangprin P, Wongsuwanlert M, Piwat S, Dahlen G, Teanpaisan R. Virulence of Aggregatibacter actinomycetemcomitans serotypes and DGGE subtypes isolated from chronic adult periodontitis in Thailand. Anaerobe. 2015;36:60–4.

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