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# Impact of *Aggregatibacter actinomycetemcomitans* on epithelial repair: in vitro study in wound model

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**ABSTRACT:** Normal wound healing occurs in four overlapping stages - hemostasis, inflammation, proliferation, and remodeling. In the oral cavity, these processes occur in an infectious environment. Among the pathogens found in the oral community, *Aggregatibacter actinomycetemcomitans* (*Aa*) constitutes a well-recognized periodontal pathogen responsible for expressing several virulence factors, which activate a host response. **Aim:** This study investigated whether *Aa*'s presence can interfere with oral keratinocyte tissue healing in an *in vitro* wound healing model. **Methods:** Two groups were defined: Group KO (n=5) and Group KO+*Aa* (n=5). The *Aa* (JP2 strains) were cultivated in anaerobiosis, and the total protein extract was obtained. The keratinocytes were cultivated with the medium of standard culture until their confluence. After confluence, plates were allocated to each group. With the pipette's tip, a "scratch" was made in the middle of each well of the plate, and the cells were cultured at 37°C in a humidified atmosphere with 5% $CO_2$. The cells received the stimulus according to groups, and, at times 0, 5, 10, 24, and 48 hours, the wound areas were visualized and standardly recorded using an inverted microscope. **Results:** When analyzing the timeframe, differences in wound measurements indicate a faster closure in the control group compared to the KO+Aa group, although not statistically significant. However, upon examining the wound closure measures, it was observed that the Aa protein extract significantly reduced wound closure at 10 and 48 hours ($p<0.05$), negatively impacting the keratinocyte's behavior. **Conclusion:** In summary, it was demonstrated that the pathogen Aa can interfere with the re-epithelization *in vitro*.

**Keywords:** Wound healing. Microbiota. Keratinocytes. HaCaT cells.

## Introduction

Normal wound healing involves four overlapping stages: hemostasis, inflammation, proliferation, and remodeling[1,2,3]. Hemostasis initiates healing by forming a blood clot that initially seals the wound. Afterward, platelets and inflammatory cells arrive at the site primarily. They provide essential functions and signals for the influx of important cells, such as fibroblasts and endothelial cells, to the injury site[1]. For healing to occur satisfactorily, restoring the intact epidermal barrier through re-epithelialization is necessary. For that, it is important to have the directed migration of keratinocytes and their proliferation and survival, as they are critical for the re-epithelialization of the wound[4]. Several diseases in the oral cavity and their treatments led to wounds that should heal under an infective condition. The oral community harbors more than 500 cultured and not-yet-cultivated species[5]. Some presented cytotoxic factors that could affect healing processes.

Among these species, some of them are well-known pathogens, presenting diverse virulence factors, as *Aggregatibacter actinomycetemcomitan* (*Aa*), *Fusobacterium nucleatum (Fn), Tannerella forsythia (Tf), Porphyromonas gingivalis (Pg), Prevotella intermedia (Pi*) and *Treponema denticola (Td)*[6]. *Aa* is a well-recognized Gram-negative anaerobic periodontal pathogen, responsible for expressing class II major histocompatibility complex molecules, the cell adhesion molecules, intercellular adhesion molecule-1, and cytokines and chemokines during inflammation[4], which activates a host response that may be associated with the pathogenesis of periodontitis[7]. However, once *Aa* can trigger a host-inflammatory response, its presence could affect healing processes. Nevertheless, little is known about the impact of pathogens on oral wound healing.

In a study investigating palate wounds for connective tissue harvesting, temporal changes in the wound area during healing were demonstrated, with alterations dependent on the surgical technique employed. Following the rupture of the mucosal barrier, oral bacteria were found to potentially impact the healing process, as observed in significant effects on wound re-epithelialization caused by *Pg* and *Fn*, identified as major contributors to the inhibition of healing[8], being the impact of *Aa* still not explored. However, a proliferative behavior of oral keratinocytes when stimulated by *Aa*-hsp60 (Heat shock protein 60) has been reported[9]. On the other hand, cytolethal distending toxin (CDT), a virulence factor of *Aa*, exhibited the potential for cell cycle arrest, cellular distension, and death[10]. *Aa* leukotoxin may also play a major role in destroying the periodontal tissues[11]. However, its impact on the wound-healing model remains unknown.

Under healthy conditions, oral polymicrobial communities still contain periodontal pathogens, although in lower proportions. However, when oral dysbiosis occurs, successive alterations in bacterial proportions and changes in the expression profile are observed, rendering them more pathogenic[5,6]. Recognizing that epithelial cells undergo alterations when challenged by periodontal pathogens, this virulent profile may potentially interfere with and delay the healing process[8], especially concerning *Aa*, one of the bacteria most strongly associated with severe periodontal disease.

Therefore, this study investigated how oral keratinocytes can modify their healing capacity when exposed to a protein extract of *Aa* serotype JP2 in an *in vitro* wound healing model.

## Materials and methods:

### Study design

To fulfill the objectives, a laboratory study was designed involving the culture of lineage cells. Cells were cultured, stimulated, and analyzed as described below.

### Groups

The following groups were defined:

- KO group (n=5): Oral keratinocyte cells derived from lineage submitted to the scratching test and cultivated in Standard medium.
- KO+*Aa* group (n=5): Oral keratinocyte cells derived from lineage submitted to the scratching test and cultivated in Standard medium addition by total *Aa* protein extract.

### *Aa* total protein extract obtention

*Aa* total protein extract was obtained as described previously[12]. Briefly, *Aa* strains (JP2) were cultivated on the surface of TSBYE agar (trypticase soy agar) (Oxoid Ltd, Basingstoke, Hampshire, England) and incubated in an atmosphere of 10% $CO_2$ (Shel Lab, Oregon, USA), at 37°C between 2 to 3 days. Grown cells as planktonic cultures were centrifuged (15min, 2°C, ≈6300g), and the culture medium was discarded. For collection and washing, 1 ml of ice-cold saline solution (NaCl 0.9%) was added to the pellet, which was resuspended. The cells were transferred to 2ml microtubes with screw caps and an o-ring (Axygen Inc., Corning Life Sciences, Union City, CA, USA). The tubes were centrifuged (4 min, 2°C, ≈13000g), and the saline solution was discarded. The cell pellet was immediately stored at -80°C until protein extraction. 700µl of ultra-pure water and ≈0.16g of 0.1mm diameter zirconia beads (BioSpec Products, Inc., Bartlesville, OK, USA) were added for total protein extraction. The microtubes were placed in a Mini-BeadBeater device (Biospec) at maximum power (3 cycles of 60 seconds with 1 min of rest on ice). Samples were centrifuged twice (8min, 4°C, ≈13000g). The bead-free supernatant was transferred to another microtube, vortexed (10s), and had the protein concentration measured using the Bradford reagent (Sigma). The homogenized extract was divided into single-use aliquots and stored at -80°C. Collections were performed on at least 3 independent cultures. The quality and integrity of the extractions were evaluated by separating 8µg of total proteins on 8.5% SDS-PAGE gels, with the aid of the Mini-Protean III system (Bio-Rad, Hercules, CA, USA), in buffer run (TBS -100mM Tris-HCl buffer, 200mM Tris, 1.37M NaCl), at 26-36mA, for 2h. Quality gels were made for all protein extracts and evaluated by Coomassie Blue. The presence of intact bands of different sizes indicated the integrity of the samples.

### Cell Viability

Cell viability was performed by the MTT method (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide). Cells were plated in 96-well plates at a $5.0x10^3$ cells/well concentration in standard medium. After 24 hours, the medium was replaced with medium containing 0,15, 0,30, 0,45, 0,60, 0,75, and 0,90 µg/ml of total *Aa* protein extract. On days 3 and 7, MTT reagent was added to each well and incubated for

4 hours at 37°C in a humidified 5% $CO_2$ incubator. At the end of the incubation period, the medium was removed, and the converted dye was solubilized with 100% ethanol. Absorbance was measured at a wavelength of 570 nm. It was decided to use the 0.45 µg/ml concentration because it was the lowest concentration that showed viability greater than 80% until day 7. The results are shown in Figure 1.

## Cell culture and Scratching test

This study used lineage human cells (HaCAT oral keratinocytes) from Periocells biobank - Periodontics Area, FOP-UNICAMP. The keratinocytes were first grown in a standard culture medium at 37°C in a humidified atmosphere with 5% $CO_2$ until confluence. Then, the cells were sown in a concentration of $1x10^5$ cells/well on 24 well plates with standard culture medium for 24 hours. After this period, the culture medium was replaced by medium containing or not the total *Aa* extract in a concentration of 0.45 µg/ml, and with the pipette's tip, a "scratch" was made in the middle of each well of the plate. Then, the cells were kept at 37°C in a humidified atmosphere with 5% $CO_2$ for up to 48 hours. Only cells between the fourth and sixth passage were used, and the experiment was replicated six times.

## Wound closure evaluation

At time 0 (immediately after the "scratch"), wound areas were visualized and recorded using an inverted microscope. The images were analyzed, and each image had its area evaluated by the ImageJ software by a calibrated single examiner (BCM, Intra-class Correlation Coefficient (ICC) > 85%). For calibration, the BCM examiner had to perform two measurements of the same scratch with an interval of 24 hours between them and obtain an ICC greater than 85%. Only wounds of similar sizes (between 900 and

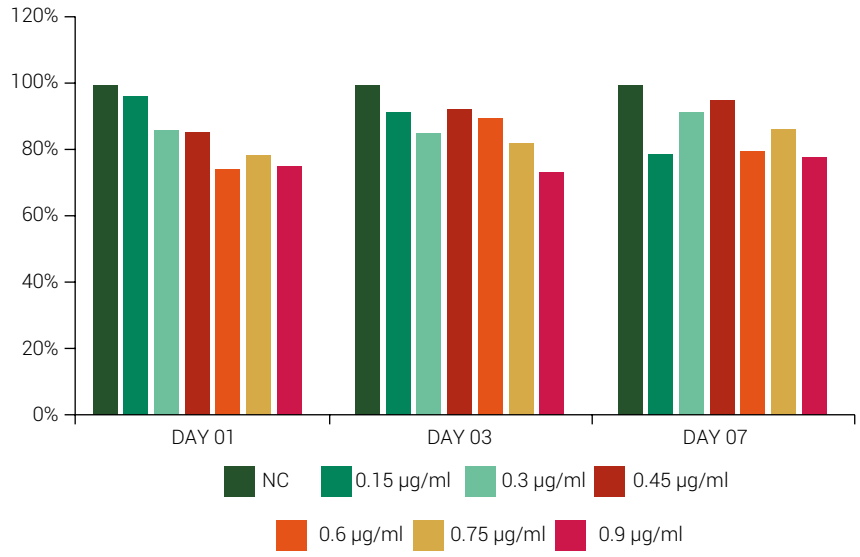


**Figure 1.** Effect of *Aa*PE on cell viability. HGFs were challenged with 0 (control) to 0,9 µg/ml of *Aa*PE (*Aggregatibacter actinomycetencomitans* protein extract), and the MTT assay for assessing cell metabolism and viability was performed on days 1, 3, and 7. The control represents 100% of viability.

1000 µm2/field) were included in the study. The heterogeneous ones were excluded. The scratch measurement was done at 0, 5, 10, 24, and 48 hours in the same plate position and region to standardize the wound healing analysis. Times were also standardized according to a previous protocol[13]. In the methodology used, wound healing or wound closure refers to the capability of epithelial cells (keratinocytes - HaCaT) to migrate to the scratch region and close the created space (wound).

### Data Analysis

Repeated measures ANOVA followed by the Tukey test was used to compare groups/times of wound measurements since it obtained a normal distribution in the Shapiro-Wilk test. A one-tailed Student's t-test was used to compare groups for the Wound closure parameter. All analyses considered 5% of significance and were done using Sigmaplot software.

## Results

As seen in Figure 2, at time 0, the wounds in the control group (NC) and the group with *Aa* were similar in size (p>0.05) and were maintained for up to 48 hours. However, when analyzing the timeframe and difference between time assessments, a difference in how the wound measures behave could indicate a faster closure in control than in the KO+*Aa* group. This phenomenon could be confirmed when analyzing wound closure (Figure 3). Standard measurements allow the determination of wound closure, considering time 0 as a reference. It is clear in Figure 3 that *Aa* protein extract reduced wound closure at 10 and 48 hours (p<0.05), negatively impacting the keratinocyte's behavior.

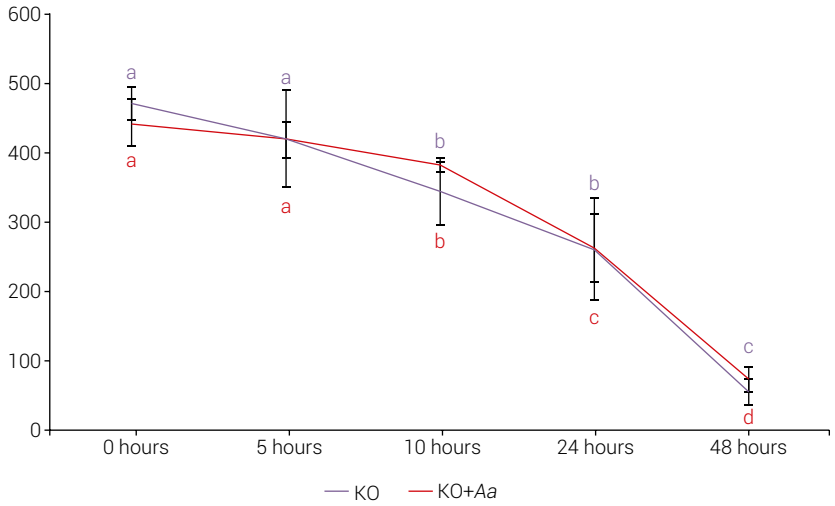


**Figure 2.** Wound measures (µm±SD) in each period. *Different letter in the same color indicates statistical differences among times within the group; No difference between groups was seen (Two-way ANOVA/Tukey, p<0.05).*

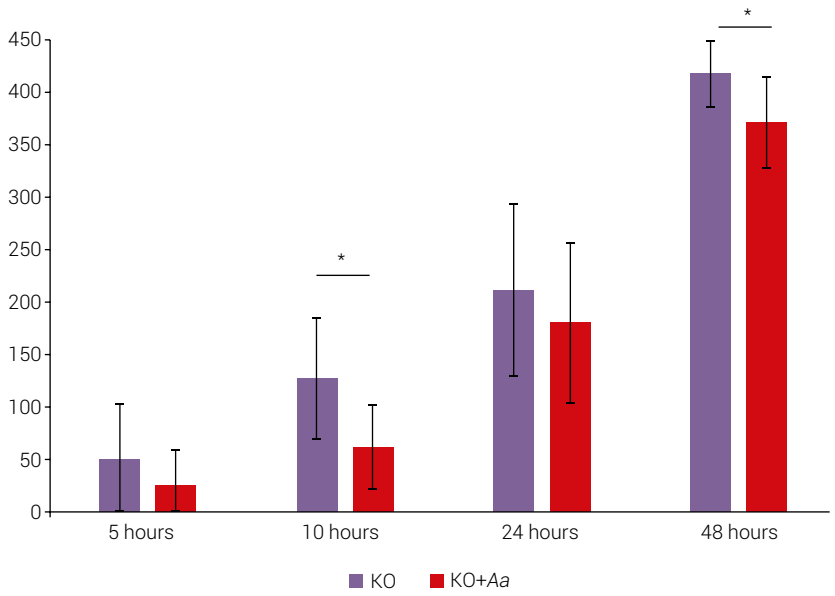


**Figure 3.** Wound closure (μm±SD) in each period.
* Indicates a statistical difference between groups (Student's t-test, p<0.05)

## Discussion

A wide variety of microorganisms inhabit the oral cavity: more than 1000 different bacterial species can colonize the mouth, and some studies carried out from the genetic sequencing of the biofilm show that approximately 500 species are similar between healthy individuals and those with periodontal diseases[14,15]. It is acceptable and credible that there is symbiotic and protective colonization of oral tissues by commensal microorganisms, which can, through different ways, act as "guardians" of periodontal health. At the same time, while simultaneously inhabiting the same niches, pathobiont microorganisms can, modulated by local and systemic conditions, participate in the installation of diseases. Oral wounds caused by disease or their treatment will heal in this environment. Thus, it is likely, although still unexplored, that bacterial agents can alter cellular events during healing. In this community, pathogens that express cytotoxic virulence factors and, thus, modulate the cellular processes involved in healing stand out. The present study showed that the presence of the protein extract of *Aa* could alter wound closure induced in keratinocyte cultures.

The healing process of oral wounds involves several cellular profiles. Keratinocytes and fibroblasts are the main constitutive cells of oral tissues and actively participate in this process[8]. Macrophages, monocytes, and neutrophils also act in the wound closure process[16] by removing necrotic tissue, cell debris, bacteria and curbing infection[17,18]. Moreover, studies confirmed that fibroblasts can be affected by different stimuli, such as hydrogen-rich water[16], hemp extract and cannabidiol[19], and L-ascorbic acid[15]. Cells involved in the hematopoietic response can also alter their response when in contact with oral pathogens, modifying cytokine production or even inducing a disease-associated pro-inflammatory response[16,17].

Studies *in vitro* assessing the role of fibroblasts in wound closure[16,18,19] observed that they present a high migration speed and proliferation during healing. However, KO is even more essential in wound healing once it is responsible for wound re-epithelialization[18] and for protecting the internal face of the wound from external stimulus while the connective tissue heals. It has been reported that keratinocytes can re-epithelialize the wound area very quickly, usually closing a wound in approximately 18 hours. This re-epithelization phenomenon enrolls keratinocytes moving into the defect 24 hours after the injury[20]. During this migration, keratinocytes from wound borders have their hemidesmosome connections dissolved from residual epithelial structures. They detach from the base of the membrane and move quickly across the wound defect. Later, as the re-epithelialization proceeds, the keratinocytes proliferate to the wound area, supporting the advancing epithelial edge. However, which cells are the first to arrive in the wound[21] is still unclear.

Meanwhile, as previously discussed, in oral wounds, the healing process will occur under an infective environment, and the type of bacteria could alter cell behavior – positive or negative. Some probiotic species, such as *Lactobacillus rhamnosus GG*, could improve wound healing, acting as a healing accelerator[20]. These results corroborate subsequent findings[13], whose two types of probiotics (*Lactobacillus casei* 324 and *Bifidobacterium pseudolongum*) accelerated the re-epithelialization of gingival epithelial cells compared to the control group.

However, pathobionts could negatively alter this process. Studies showed that when these cells are exposed to *Pg*, their proliferation capacity drops considerably. *Pg* can inhibit tissue growth[8,18] and display a destructive effect[13]. When OBA-9 (immortalized human gingival epithelial cells) are infected with *Pg* W83, the wound becomes larger instead of lessening by reducing the cell viability compared to the control group[13]. Besides, keratinocytes can present healing delayed by the influence of two oral pathogens (*Pg* and *Fn*), and it was noticed that the long-term effect (especially in days 4 and 8) caused a major delay in the healing in at least 70%[8].

In our study, we evaluate the effect of total protein extract from *Aa* serotype JP2, which also has a destructive effect. *Aa*-JP2 is the most cytotoxic serotype and a highly pathogenic species because its highly leukotoxic strains can produce 10 to 20-fold more toxin than the others[22]. It is well known that *Aa*-JP2 interferes with the host's innate immune defense and response[11,12]. Studies with oral cells showed that this could lead to an imbalanced inflammatory response[12], which could affect healing, as observed in the study. All this can disrupt normal periodontal tissue remodeling processes, ultimately promoting collateral tissue damage, which explains the delay in healing[22]. However, although potentially cytotoxic, up to now, there has been no study assessing the impact of *Aa* on wound healing. The present study shows that it negatively affected the healing process since lower wound closure could be seen when keratinocytes were cultivated with total *Aa* protein.

One factor that may explain why *Aa* decreases healing is that it disrupts epithelial integrity, facilitates bacterial invasion into deeper tissues, and subsequently destroys periodontal connective tissue[23]. Disturbances caused by the cell-cell junction's stability and dynamics can affect the epithelial layers' barrier properties, harm tissue remodeling throughout development, and impair the healing compared to healthy

tissue[23]. *Aa* can act on tissue destruction and delay healing. A study demonstrated that *Aa* secretes a cytolethal distending toxin that likely permeates the widened interstitial spaces of the junctional epithelium and in non-keratinized forms of gingival epithelium to affect their basal cell layers[24]. The distension of human gingival epithelial cells and the dissolution of cell junctions may promote broader penetration of the toxin throughout the tissue, which may signal the recruitment of inflammatory cells, consequently increasing the extent of tissue damage due to the production of inflammatory mediators. This study showed a significant difference at two follow-up times (10 and 48 hours). On the other hand, no statistical difference could be identified after 5 and 24 hours of exposure. Epithelial cells are the first to arrive at the wound site, and the epithelial healing process is expected to occur rapidly[20]. There is a response time for keratinocytes to the *Aa* protein extract (*Aa*PE) exposure. After exposure to *Aa*PE, the cell recognizes the pathogenic protein through TLRs. Its metabolic activity begins to be modulated, generating a lack of production or defective production of proteins responsible for cell junctions[23]. This entire process can take hours to occur. This is the plausibility of the absence of difference in the cell response after 5 hours in this study. After this process, cellular activity is damped by *Aa* toxins, slowing down the epithelial repair, which explains the later closure response for the test group (48 hours).

The results achieved in this study elucidate a slight portion of the etiopathogenic aspects of periodontal disease. In periodontitis, the dysbiotic presence of periodontopathogens such as *Aa* and *Pg* at subgingival biofilm harms gingival tissue through the bacterial adherence to the epithelial cells, which colonizes the connective tissue, stimulating the cells to secrete cytokines responsible for recruiting inflammatory cells by inducing changes in signaling pathways and gene expression[25]. This methodology used *Aa*PE for keratinocyte stimulation because of its proximity to periodontitis. Although there are six serotypes of *Aa* (a–g), serotype b (JP2 genotype) is the one that is associated with a greater risk of periodontal insertion loss[22]. In our study, the total protein of this more cytotoxic strain was used, and several virulent aspects were given to cells. Given the results obtained, in addition to being a trigger for the onset of the disease, the presence of Aa can also alter tissue healing after initial damage or periodontal instrumentation. This way, clinicians must focus more on controlling subgingival plaque and dysbiosis after periodontal treatment and other procedures involving periodontal tissues and re-epithelization, *e.g.*, aesthetic crown lengthening.

In this study, we assessed that wound healing could indeed be influenced by a bacterial pathogen such as *Aa*; however, this study presented some limitations: The lack of an *in vivo* study to see how the subgingival microbiota and a complete biofilm with a larger number of pathogens, as in periodontitis, can affect oral tissue healing, and the lack of another cell type, such as fibroblasts, for greater comparison effect in the results. Therefore, it would be necessary to include at least one more pathogen and cell type for a better *in vitro* study and to evaluate the possibility of carrying out additional *in vivo* studies to determine the impact of oral bacteria on wound healing *in vivo*.

In conclusion, the pathogen *Aggregatibacter actinomycetemcomitans* can interfere with wound closure by keratinocytes. This may be due to disturbances caused by the stability and dynamics of the cell-cell junction caused by *Aa*.

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## Conflict of interest

The authors have no conflict of interest to disclose.

## Data availability

Datasets related to this article will be available to the corresponding author upon request.

## Author contribution

**Bianca Carvalho Mendes:** Data collection, Writing the manuscript. **Camila Schmidt Stolf:** Conception and design of the study, Data analysis, Writing the manuscript. **Hélvis Enri de Sousa Paz:** Review and Editing, Writing the manuscript. **Letícia Sandoli Arroteia:** Data analysis, Interpretation and Statistics. **Lucas de Paula Ramos:** Data collection, Interpretation and Statistics. **Mauro Pedrine Santamaria:** Data collection, Interpretation and Statistics. **Karina Gonzales Silvério Ruiz:** Conception and design of the study, Review and Editing. **Mabelle de Freitas Monteiro:** Writing the manuscript, Review and Editing. **Renato Corrêa Viana Casarin:** Conception and design of the study, Writing the manuscript, Review and Editing. We declare that all authors actively participated in the manuscript findings, revised and approved the final version of the manuscript.

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