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RESEARCH ARTICLE

Bacterial short-chain fatty acid metabolites modulate the inflammatory response against infectious bacteria

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Abstract

Short-chain fatty acids (SCFAs), predominantly acetic, propionic, and butyric acids, are bacterial metabolites with an important role in the maintenance of homeostasis due to their metabolic and immunomodulatory actions. Some evidence suggests that they may also be relevant during infections. Therefore, we aimed to investigate the effects of SCFAs in the effector functions of neutrophils to an opportunistic pathogenic bacterium, *Aggregatibacter actinomycetemcomitans*. Using a subcutaneous model to generate a mono, isolated infection of *A. actinomycetemcomitans*, we demonstrated that the presence of the SCFAs in situ did not affect leukocyte accumulation but altered the effector mechanisms of migrating neutrophils by downregulating the production of cytokines, their phagocytic capacity, and killing the bacteria, thus impairing the containment of *A. actinomycetemcomitans*. Similar effects were observed with bacteria-stimulated neutrophils incubated with SCFAs in vitro. These effects were independent of free-fatty acid receptor 2 (FFAR2) activation, the main SCFA receptor expressed on neutrophils, occurring possibly through inhibition of histone deacetylases because similar effects were obtained by using histone deacetylase inhibitors, such as SAHA, MS-275, and RGFP 966. Considering the findings of this study, we hypothesized that in an infectious condition, SCFAs may exert a detrimental effect on the host by inhibiting neutrophil's effector functions.

KEYWORDS

Aggregatibacter actinomycetemcomitans, anaerobic bacteria, butyrate, histone deacetylases, neutrophils, propionate

1 | INTRODUCTION

Short-chain fatty acids (SCFAs) are bacterial metabolites present at high concentrations in mucosal surfaces at different locations, including the oral cavity (1–16 mM), the intestinal tract (70–140 mM), and female genital organs (0.3–30 mM; Chaudry et al., 2004; Lu, Meng, Gao, Xu, & Feng, 2014; Macfarlane & Macfarlane, 2003; Qiqiang, Huanxin, & Xuejun, 2012). Different species of bacteria collectively contribute to the production of acetic, propionic, and butyric acids, the most abundant SCFAs, at these sites through fermentation of saccharides and host components, such as mucin and proteins (Cook & Sellin, 1998). These bacterial metabolites are essential elements in the interaction between microbiota and the host. Indeed, associations between changes in their production and the development of immune-mediated inflammatory disorders (e.g., insulin resistance, colitis, and asthma) have been identified (as reviewed by Ferreira et al., 2014).

Immune cells are important targets of SCFAs, which act on them through different mechanisms, including activation of G protein-coupled receptors (GPCRs; i.e., FFAR2, FFAR3, GPR109a, and Olfr78) and inhibition of histone deacetylases (HDACs). Leukocyte recruitment and effector functions are modulated by SCFAs (revised by Corrêa-Oliveira, Fachi, Vieira, Sato, & Vinolo, 2016). These metabolites can either potentiate or attenuate inflammatory and immune responses depending on different conditions. Examples of this paradox are the findings that SCFAs can either increase or decrease neutrophil recruitment in vivo (Kim, Kang, Park, Yanagisawa, & Kim, 2013; Vinolo, Rodrigues et al., 2011) or even induce both effector (Th1 and Th17) and regulatory T cells (Park, Goergen, HogenEsch, & Kim, 2016).

Considering that SCFAs present relevant immunomodulatory effects, it is not a surprise that some studies have suggested their participation in the initiation and progression of infectious diseases (Al-Mushrif, Eley, & Jones, 2000; Niederman, Buyle-Bodin, Lu,

Robinson, & Naleway, 1997; Niederman, Zhang, & Kashket, 1997). Individuals with periodontitis, a chronic inflammatory disease that affects the integrity of the tooth-supporting tissues (Hajishengallis, 2015), present significant changes in local SCFA concentrations. Qiqiang et al. (2012) reported higher concentrations of propionic (11.68 ± 8.84 vs. 5.87 ± 3.35 mM) and butyric acids (3.11 ± 1.86 vs. 1.10 ± 0.87 mM) in the gingival crevicular fluid of individuals with chronic periodontitis compared to healthy individuals. Higher concentrations of acetic, propionic, and butyric acids were also observed in individuals with aggressive periodontitis (26.0 vs. 11.3, 8.8 vs. 2.1, and 2.5 vs. 0.0 mM, respectively; Lu et al., 2014). Interestingly, the treatment of these conditions is associated with drastic reductions in SCFA concentrations (Chaudry et al., 2004; Lu et al., 2014; Qiqiang et al., 2012). These facts can be in part explained by considering the local microbiota. A comparison of the oral microbiota metatranscriptome between healthy individuals and patients with periodontal disease revealed changes in genes involved in bacterial metabolism during disease progression, including enzymes that participate in the production of SCFAs (Jorth et al., 2014). Together, these and other studies demonstrate that changes in SCFA production occur in these infectious conditions. However, a question that remains to be addressed is whether these changes interfere with the immune response and disease outcome or are merely a consequence of the changes in microbial components that accompany the pathological condition with no specific relevant effects to the system. Moreover, even with this possible effect, it is also unclear if these metabolites, once produced, facilitate the spread of pathogens or help the immune system to control the infection.

In this regard, the aim of this study was to investigate the effects of SCFAs in the immune response to *Aggregatibacter actinomycetemcomitans*, a Gram-negative, facultative anaerobic bacteria associated with localized aggressive periodontitis and other nonoral infections in humans, such as endocarditis, pericarditis, infectious arthritis, and various types of abscesses (van Winkelhoff & Slots, 1999). We used a subcutaneous chamber model of infection (Genco, Cutler, Kapczynski, Maloney, & Arnold, 1991), which allowed us to investigate the effects of a mixture of acetate, propionate, and butyrate at similar concentrations to those found at sites of infection (Lu et al., 2014) in the immune response to the isolated bacterium. In summary, this chamber model system permits the generation of an isolated compartment in an animal, although still permissive to the immune system, in which we can initiate and maintain a monoinfection of *A. actinomycetemcomitans*. In addition to these in vivo experiments, we also performed in vitro analysis using murine and human neutrophils to explore the molecular mechanisms involved in the SCFA interactions with the cells, including activation of FFAR2, the main GPCR activated by SCFAs in neutrophils, and HDAC inhibition.

2 | RESULTS

2.1 | Short-chain fatty acids do not alter leukocyte migration to the infectious site

Short-chain fatty acids per se induce neutrophil migration in vivo and in vitro (Maslowski et al., 2009; Sina et al., 2009; Vinolo et al., 2009; Vinolo, Ferguson et al., 2011). Therefore, we first analysed the recruitment of cells to the subcutaneous chamber at three distinct time points (4, 24, and 72 hr) after bacteria inoculation in the chamber. As shown in Figure 1, the inoculation of *A. actinomycetemcomitans* (1×10^8 colony-forming units [CFUs]/100 μl), alone or in combination with SCFAs, induced a massive accumulation of cells, predominantly polymorphonuclear neutrophils, at the chamber. Increased by 3.6, 9.6, and 4.5 times, the total number of cells was found after 4, 24, and 72 hr, respectively, of bacteria inoculation in comparison to the negative condition (NC; phosphate-buffered saline [PBS] inoculation). Leukocyte accumulation in response to *A. actinomycetemcomitans* presented similar kinetics, and it was quantitatively the same regardless of the presence or absence of SCFAs (Figure 1).

As shown in Figure 1d, inoculation of *A. actinomycetemcomitans* in the chamber caused a self-limited response characterized by maximum leukocyte infiltration at 24 hr followed by a decline in their numbers after 72 hr. In all the analysed periods, the predominant cells were neutrophils, but a significant percentage of mononuclear cells, including lymphocytes and mainly macrophages, were also observed (Figure 1d). Experiments in which the SCFAs were inoculated in the chamber in the absence of bacteria were also performed, but there was still no effect on leukocyte numbers (Figure 1c). Taken together, these results suggest that SCFAs, at the concentrations found in infectious sites, in opposition to their effect in sterile models of inflammation, do not affect neutrophil accumulation.

2.2 | SCFAs decreased the phagocytic capacity of neutrophils, leading to impairment in the containment of *A. actinomycetemcomitans*

Next, we analysed the amount of viable bacteria in the chamber exudates. A sample of the exudates collected 4 and 24 hr after bacteria inoculation was serially diluted and plated in brain heart infusion (BHI) chocolate agar plates. The number of CFUs was counted 3–4 days later. We observed that the number of bacteria in the chambers (originally 1×10^8 CFUs) rapidly decreased after their inoculation in vivo: A reduction of more than 90% of the viable bacteria was observed after only 4 hr of inoculation. In these experiments, no signs of systemic dissemination of the bacteria were observed. These findings suggest that, in this model of infection, *A. actinomycetemcomitans* causes a localized and self-limited response.

In the presence of SCFAs, the amount of bacteria in the chamber was higher for both the 4 hr (~3 times higher) and 24 hr (~10 times higher) groups when compared to the control group (Figure 2a and 2b). This effect of SCFAs was not associated with a cytotoxic effect of these molecules on inflammatory cells because few cells presented loss of membrane integrity or phosphatidylserine externalization in the conditions used in vivo (Figure S1).

Next, we investigated if the SCFAs directly affected bacterial growth, an effect that could account for the results observed in vivo. We cultivated the bacteria in liquid BHI in the presence or absence of SCFAs at the same concentrations used in vivo. The optical density of the culture for over 48 hr and the number of CFUs (from plating the 24-hr samples) were analysed, but no significant effect of the SCFAs was observed (Figure S2).

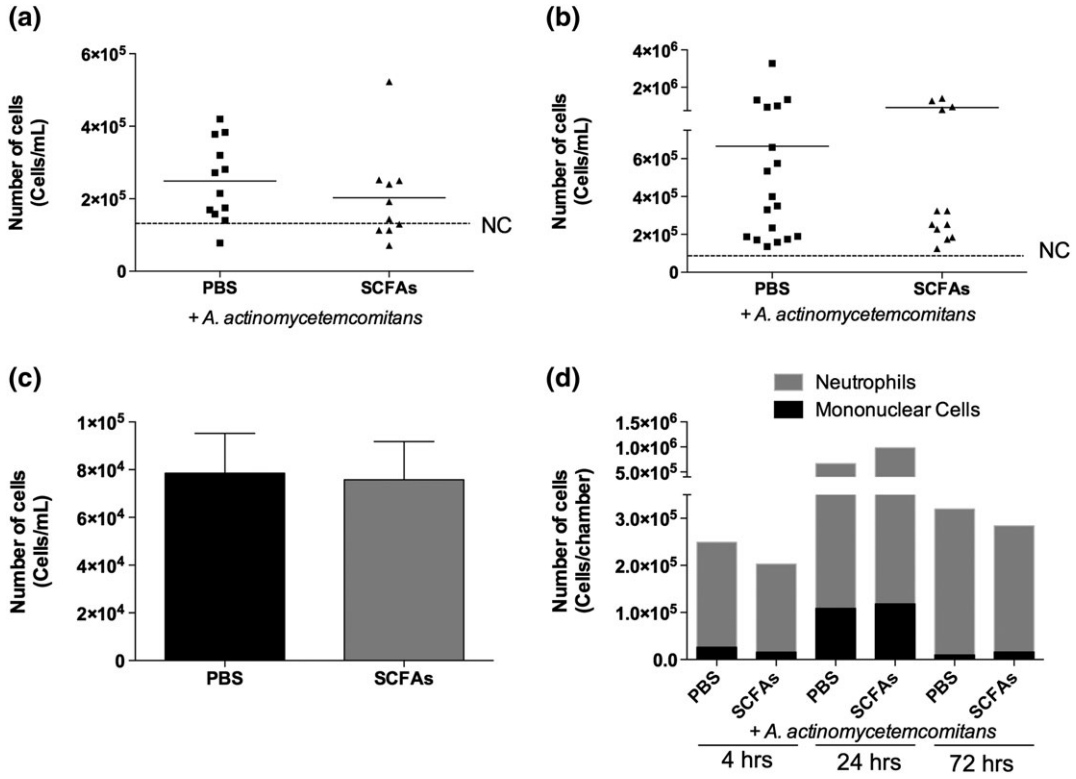

FIGURE 1 Short-chain fatty acids (SCFAs) do not modify leukocyte migration in vivo in response to *Aggregatibacter actinomycetemcomitans*. Leukocyte recruitment 4 (a) and 24 hr (b) after inoculation of *A. actinomycetemcomitans* combined or not with SCFAs. In the graphs, each square or triangle represents an animal of five distinct experiments for both times. The horizontal bars represent the average of each group (N = 10–18 animals per group). The dashed line indicates the mean value obtained for the negative control group (NC, animals that were inoculated with phosphate-buffered saline [PBS]). The results were analysed by the Mann–Whitney test, and significance was considered for $p < .05$. (c) Leukocyte recruitment 4 hr after inoculation with PBS or SCFAs without bacteria. (d) Profiles of leukocyte migration over time for each condition. For all graphs, SCFAs represents a mix of 26 mM acetate, 10 mM propionate, and 2.5 mM butyrate

Considering these results, we next tested whether the SCFAs modulate neutrophil phagocytosis of *A. actinomycetemcomitans* in vivo. *A. actinomycetemcomitans* labelled with pHrodo succinimidyl ester, a pH-sensitive fluorescent dye, which is very useful for investigating phagocytosis (Simons, 2010), and cells marked with anti-Ly6G were analysed by flow cytometry. Bacteria were inoculated in the chamber, and the inflammatory exudate was collected 4 hr after the inoculation, a sufficient period for observing phagocytosis in vivo. In this experiment, the presence of SCFAs caused a significant reduction in the capacity of neutrophils to internalize *A. actinomycetemcomitans* in comparison to the control condition (Figure 2c and 2d).

Given the in vivo data, we next aimed to investigate the effect of acetate, propionate, and butyrate in isolated neutrophils in vitro. In accordance with the results reported above, we observed a significant reduction in the phagocytosis of serum-opsonized *A. actinomycetemcomitans* by neutrophils in vitro (Figure 3), an effect that was also observed with green fluorescent protein-expressing *Escherichia coli* (Figure 3c). Using FFAR2-deficient cells, we found that the effect in the phagocytosis of bacteria was independent of FFAR2 activation by SCFAs (Figure 3b). Additionally, we found that incubation of human neutrophils with the SCFA mixture used in the in vivo experiments also reduced their capacity to phagocytose (Figure 3e). Taken together, these results indicate that SCFAs impair bacterial phagocytosis through a FFAR2-independent mechanism, reducing the in vivo microbicidal activity of neutrophils.

2.3 | SCFAs modulate the production of inflammatory mediators in vivo and in vitro

To further investigate the effect of SCFAs in the in vivo response to *A. actinomycetemcomitans*, we next measured inflammatory mediators, including proinflammatory cytokines (tumour necrosis factor-α [TNF-α], interleukin [IL] 1β, IL-6, and IL-12), chemokines (Cxcl1 and Cxcl2), and IL-10, an antiinflammatory cytokine, in the exudates collected from the chambers at 4 and 24 hr after bacteria inoculation. As expected, 4 hr after the inoculation of *A. actinomycetemcomitans*, increased concentrations of cytokines and chemokines were observed: an approximately 2,000-fold increase for TNF-α, IL-6, and Cxcl1, a 100-fold increase for IL-10, and a 25-fold increase for IL-1β and Cxcl2 when compared to the control group without the bacteria (Figure 4a–c and Figure S3).

The amount of inflammatory mediators in the chamber rapidly decreased after 24 hr in comparison to 4 hr (Figure 4a–c and Figure S3), with the exception of IL-12, which levels were very low at both analysed points. When comparing the groups with or without SCFAs, no significant effect was observed at 4 hr. However, at 24 hr, the presence of SCFAs led to a significant reduction in the concentrations of TNF-α (Figure 4a), Cxcl2 (Figure 4b), and IL-10 (Figure 4c). This effect was not observed for IL-1β, IL-6, IL-12, or Cxcl1 (Figure S3), indicating that it is not an unspecific or general effect of SCFAs.

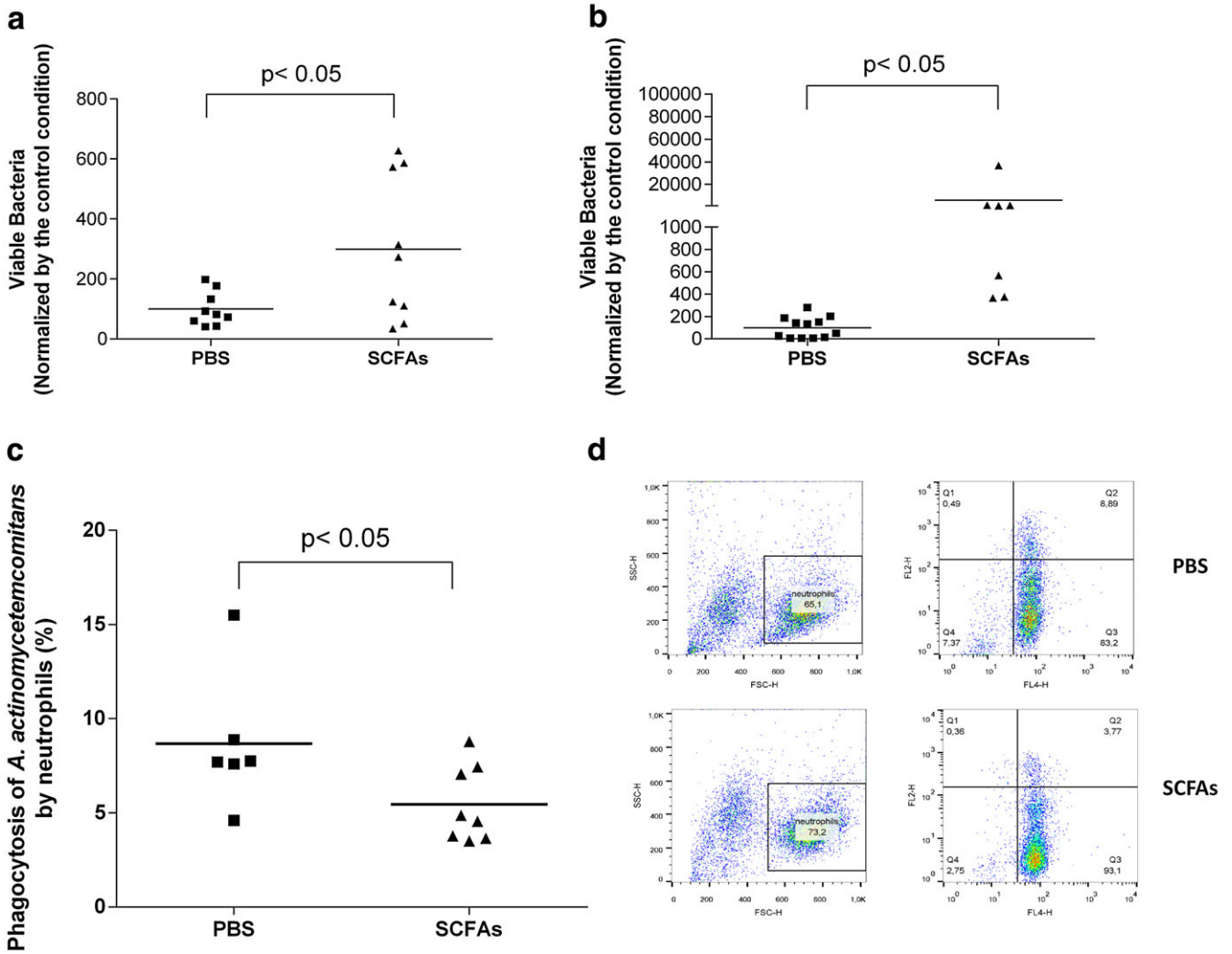

FIGURE 2 Short-chain fatty acids (SCFAs) impair the killing of *Aggregatibacter actinomycetemcomitans* in vivo. The exudate collected after 4 (a) or 24 hr (b) of *A. actinomycetemcomitans* inoculation in the chamber was diluted and plated in brain heart infusion chocolate agar. Bacterial colonies were counted, and the number of colony-forming units obtained for each abscess was normalized by the values obtained in the control condition (inoculation of *A. actinomycetemcomitans* alone). In the graphs, each symbol represents an animal. The horizontal bars represent the average of each group (*N* = 7–12 animals per group). Phagocytosis of *A. actinomycetemcomitans* by neutrophils was analysed in vivo (c and d). pHrodo-marked bacteria were inoculated in the chamber. Four hours later, the number of neutrophils (Ly6G positive cells) with pHrodo-marked bacteria was analysed using a flow cytometer. In the graphs, each symbol represents an animal. The horizontal bars represent the average of each group (*N* = 6–8 animals per group). The results were analysed by the Mann–Whitney test. Representative flow data showing phagocytosis of *A. actinomycetemcomitans* in vivo by neutrophils in the presence or absence of SCFAs (d). For all graphs, SCFAs represents a mix of 26 mM acetate, 10 mM propionate, and 2.5 mM butyrate. PBS = phosphate-buffered saline

As previously described, neutrophils are the predominant leukocytes present at the inflammatory site after inoculation of *A. actinomycetemcomitans* at the time points analysed in this study. In this context, these cells are likely the main sources of cytokines and other inflammatory mediators, which can regulate their own effector functions and the recruitment and activation of other cells. Previous studies have found that SCFAs modify the production of cytokines by human and rodent neutrophils stimulated with toll-like receptor (TLR) agonists. Given the in vivo findings and the fact that no study has investigated the effect of SCFAs in the presence of bacteria, we next examined whether SCFAs had a direct effect on cytokine production by *A. actinomycetemcomitans*-stimulated neutrophils. To examine this, we collected thioglycollate-elicited neutrophils and incubated them in the presence of nontoxic concentrations of SCFAs and *A. actinomycetemcomitans*. First, we evaluated the production of cytokines by cells incubated with the same mixture of SCFAs used in vivo but diluted five (1/5) or 20 times (1/20) in media for 6 hr. The SCFAs (diluted 1/5) reduced the production of TNF-α and IL-10 by neutrophils stimulated with *A. actinomycetemcomitans* while inducing the opposite effect for IL-1β (Figure 4d). Additionally, we also found a significant reduction in TNF-α production by *A. actinomycetemcomitans*-stimulated human neutrophils incubated with the different SCFA dilutions (Figure 4e).

Next, we analysed the individual effect of different concentrations of acetate, propionate, and butyrate on the cytokine production of neutrophils. In the presence of propionate (8 mM) or butyrate (1.6 and 3.2 mM), a significant reduction in the production of TNF-α and IL-10 was observed by *A. actinomycetemcomitans*-stimulated

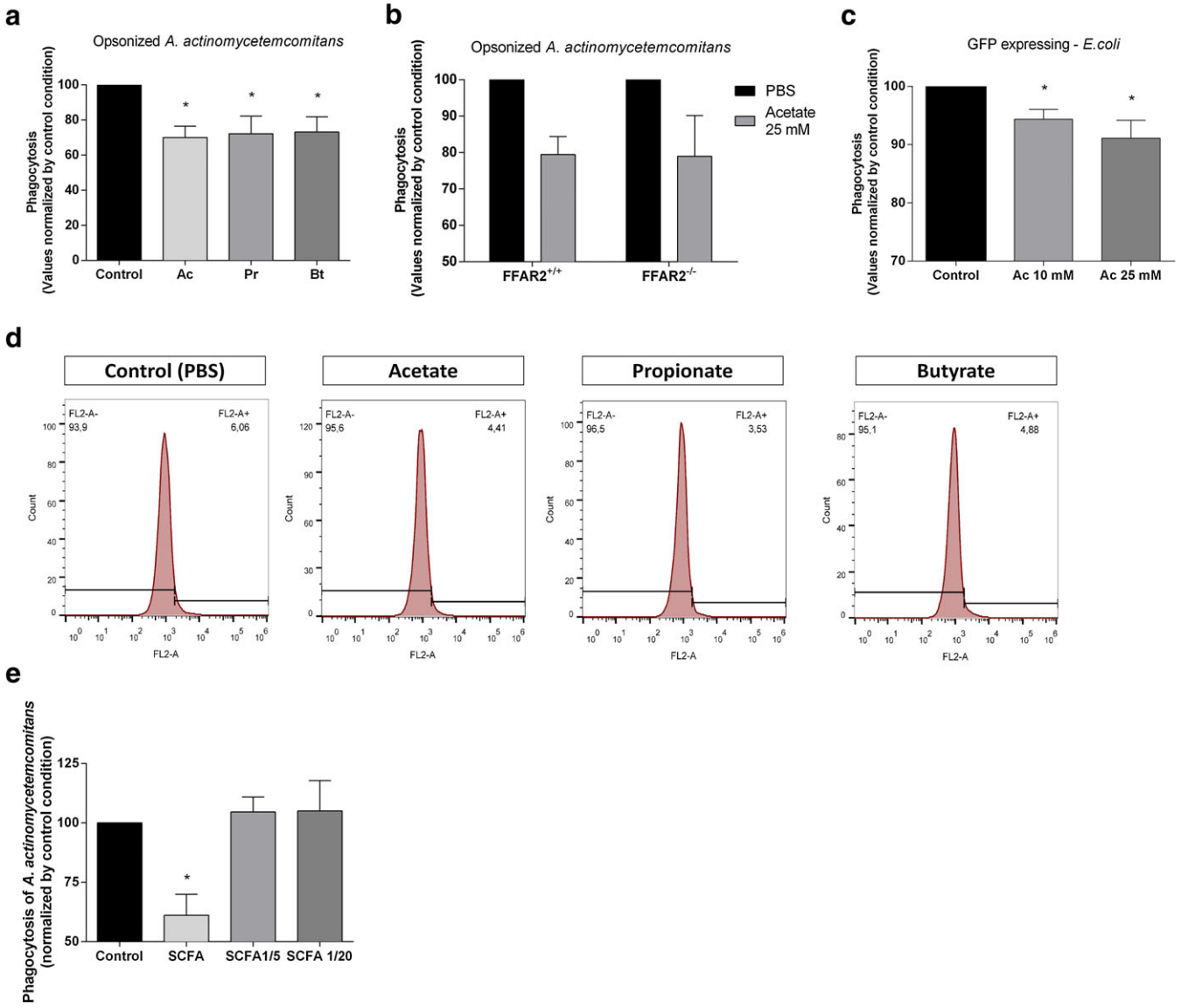

FIGURE 3 Short-chain fatty acids (SCFAs) reduce the phagocytosis of bacteria by neutrophils through an FFAR2-independent mechanism. Neutrophils were incubated with pHrodo-marked bacteria, previously opsonized, in the presence of SCFAs for 2 hr (control = phosphate-buffered saline [PBS], Ac = acetate 25 mM, Pr = propionate 8 mM, and Bt = butyrate 3.2 mM). Phagocytosis was then analysed by flow cytometry (a). *N* = 6 animals. Phagocytosis assay was performed with FFAR2$^{+/+}$ and FFAR2$^{-/-}$ cells incubated with *A. actinomycetemcomitans* and acetate 25 mM (b). *N* = 4–8 animals per group. Neutrophils were incubated with *Escherichia coli* expressing green fluorescent protein (GFP), previously opsonized, in the presence of acetate (10 or 25 mM) for 2 hr. Phagocytosis was then analysed by flow cytometry (c). *N* = 4 animals. Representative figures are presented for each examined condition (d). The percentage of positive cells obtained in the experiment is described in Figure 3a. Human neutrophils were incubated with pHrodo-marked bacteria, previously opsonized, in the presence of different dilutions of a mixture of SCFAs for 2 hr. Phagocytosis was then analysed by flow cytometry (e). *N* = 5 samples. All the results were normalized by control values (C = 100%) and are presented as the mean ± SEM. $^{*}p < .05$ compared to the control condition

neutrophils (Figure 5a and 5c). On the other hand, there was an increase in the production of IL-1β (Figure 5b), and no effect was observed for Cxcl1 production (Figure 5d). No effects were observed for acetate at any concentration. When neutrophils were incubated with lipopolysaccharide (LPS) instead of *A. actinomycetemcomitans*, a similar pattern of response to SCFA treatment was obtained (Figure S4).

To confirm these results, we repeated the experiment using a highly purified population of neutrophils (>85%). For that, bone marrow cells were collected and submitted to negative sorting, as previously described (Hasenberg et al., 2011). The results obtained with these cells, stimulated with both *A. actinomycetemcomitans* and LPS, confirmed the findings in the elicited neutrophils (Figure S5). Additionally, we analysed the messenger RNA (mRNA) expression in the neutrophils isolated from the bone marrow and stimulated with *A. actinomycetemcomitans* in the presence or absence of butyrate, the most potent SCFA regarding the effects on cytokine production. In support of the other results, a marked reduction in the expression of TNF-α and to lesser extent in IL-10 was found in *A. actinomycetemcomitans*-stimulated neutrophils incubated with

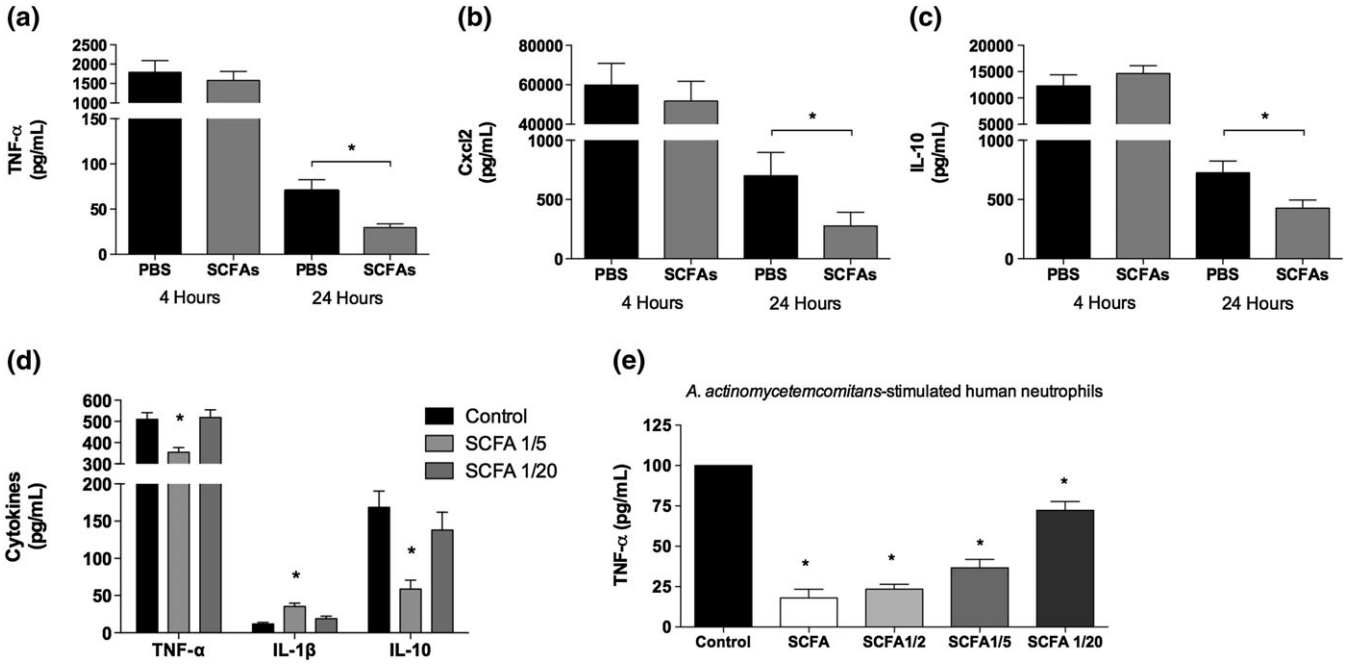

FIGURE 4 Short-chain fatty acids (SCFAs) alter the production of cytokines in vivo and in vitro. Inflammatory mediators (tumour necrosis factor [TNF] α, Cxcl2, and interleukin [IL] 10) were measured, by ELISA, in exudates obtained from the chambers 4 and 24 hr after inoculation of *Aggregatibacter actinomycetemcomitans*. *N* = 7–12 animals for the time of 4 hr and 10–18 animals for the time of 24 hr (a–c). Murine neutrophils were incubated for 6 hr in the presence of different dilutions of a mixture of SCFAs (26 mM acetate, 10 mM propionate, and 2.5 mM butyrate) and *A. actinomycetemcomitans* (multiplicity of infection 10:1). The concentrations of TNF-α, IL-1β, and IL-10 (d) were determined in the culture supernatants. *N* = 4–5 animals. Human neutrophils were incubated for 6 hr in the presence of different dilutions of the same mixture of SCFAs cited above and *A. actinomycetemcomitans*. TNF-α (e) was determined in the culture supernatants. *N* = 5 samples. All the results are presented as the mean ± SEM. **p* < .05 compared with the control condition. PBS = phosphate-buffered saline

butyrate in comparison to the control condition (without butyrate). As opposed to the effect described for the protein quantification, IL-1β mRNA expression was also reduced in the cells treated with butyrate, although no effect was observed for TLR4 or inducible nitric oxide synthase expression, which remained stable after stimulation with *A. actinomycetemcomitans* (the dashed line in the graphs represents the results with cells not stimulated, Figure 5e).

One of the mechanisms by which SCFAs modulate cytokine production by the cells, such as epithelial cells, neutrophils, and macrophages, is through activation of GPCRs (Kim et al., 2013; Singh et al., 2014) such as FFAR2, which is highly expressed in neutrophils. However, when we analysed the effect of the SCFAs in the elicited neutrophils from $FFAR2^{-/-}$ mice stimulated with *A. actinomycetemcomitans*, the response pattern was similar to the wild-type mice ($FFAR2^{+/+}$ mice), indicating that the SCFA effect on the production of cytokines by these cells is independent of this molecular pathway (Figure 6).

2.4 | SCFAs might act on neutrophils through inhibition of HDACs

Short-chain fatty acids are pan-inhibitors of HDACs (HDACis), targeting classes I (HDACs 1, 2, 3, and 8); II (HDACs 4, 5, 6, 7, 9, and 10); and IV (HDACs 11) HDACs. The inhibition of HDACs is associated with an increase in protein acetylation. Here, we found that butyrate, but also to a lesser extent the other SCFAs at the concentrations used in the in vitro assays, substantially increased the content of acetylated histone H3 lysine 9 (H3K9ac) in the neutrophils after 2 hr of incubation (Figure 7a). To investigate the possibility that the inhibition of HDACs was involved in the effects of SCFAs in cytokine production and in the phagocytosis of bacteria, we repeated these analyses using a pan-inhibitor of HDAC (SAHA) and isoform-selective HDACis (MS-275 for isoforms 1 and 3; CI994 for isoform 1; PCI-34051 for isoform 8; and RGF966 for isoform 3), and we compared the results to the data obtained with the SCFAs.

Neutrophils incubated with CI994, MS-275, or RGFP966 and stimulated with *A. actinomycetemcomitans* presented a reduction in TNF-α production (Figure 7b) and an increase in IL-1β (not significant for RGFP966; Figure 7c). For IL-10, no effect of the HDACis was observed (Figure 7d). Similar results were found for TNF-α and IL-1β modulation when the cells were incubated with LPS instead of *A. actinomycetemcomitans*. However, in this latter experiment, there was also an increase in IL-10 production by CI994, MS-275, or RGFP966 (Figure S6).

In addition to the production of cytokines, we also tested if HDACis affect the phagocytosis of opsonized *A. actinomycetemcomitans*. In this latter experiment, we found that RGFP 966, MS-275, and SAHA, but not the other compounds, reduced the phagocytosis of the bacteria (Figure 7e). In conclusion, these results indicate that SCFAs inhibit HDAC activity in neutrophils and that this mechanism may be involved in some of their actions on the response of neutrophils to *A. actinomycetemcomitans*. Based on the findings with the HDACis, we suggest that inhibition of the HDAC isoforms 1 and 3, but not 8, may play a role in the effects of SCFAs in the response of neutrophils to bacteria.

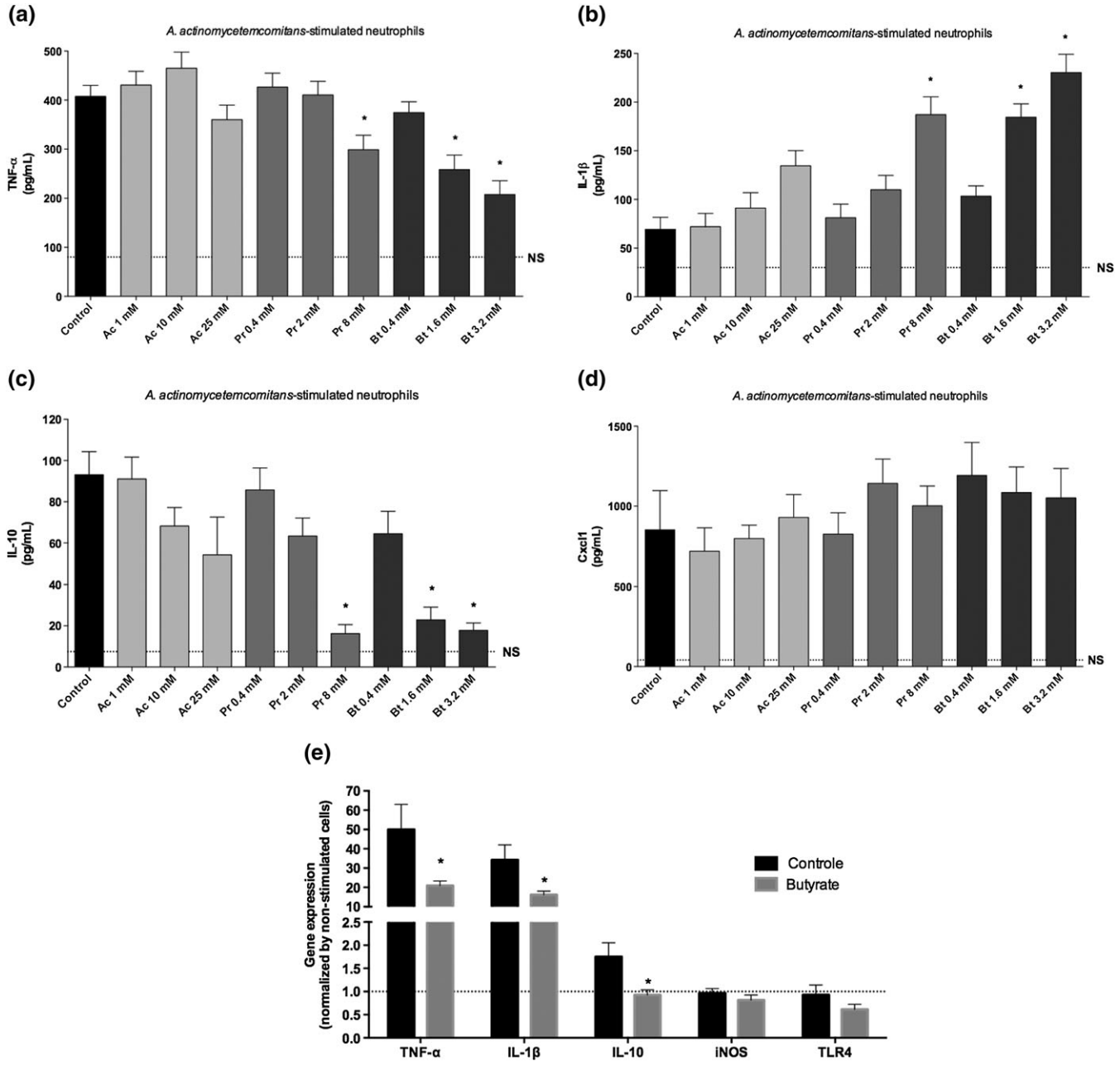

FIGURE 5 Propionate and butyrate modulate the production of cytokines in vitro. Neutrophils were incubated for 6 hr in the presence of isolated short-chain fatty acids (Ac = acetate, Pr = propionate, and Bt = butyrate) and *Aggregatibacter actinomycetemcomitans* (multiplicity of infection 10:1). The concentrations of tumour necrosis factor (TNF) α (a), interleukin (IL) 1β (b), IL-10 (c), and Cxcl1 (d) were determined in the culture supernatants by ELISA. *N* = 6–8 animals. The dashed lines in the graphs indicate the mean value obtained for not-stimulated cells (NS). Expressions of TNF-α, IL-1β, IL-10, iNOS, and toll-like receptor (TLR) 4 messenger RNA by *A. actinomycetemcomitans*-stimulated neutrophils were analysed in the presence of 3.2 mM butyrate (e). *N* = 2 animals in duplicate. The dashed line in the graph indicates the mean value obtained in NS cells. All the data are reported as the mean ± SEM. **p* < .05 compared with the control condition

3 | DISCUSSION

In this study, we demonstrated that the presence of SCFAs in the infectious site attenuates the immune response to *A. actinomycetemcomitans*. SCFA-modified effector functions of neutrophils include phagocytosis and cytokine production in response to bacteria. Despite the fact that the molecular pathway is not described, we present results indicating that the inhibition of specific isoforms of HDACs, namely, HDAC 1 and 3, but not activation of FFAR2, is involved in the effects of SCFAs in neutrophils.

Short-chain fatty acids are bacterial metabolites produced in the intestinal tract as end products of dietary fibre fermentation. Initially described as fuel molecules for epithelial cells, hepatocytes, and peripheral tissues (Pomare, Branch, & Cummings, 1985), they are now associated with important immunomodulatory effects (reviewed by Corrêa-Oliveira et al., 2016). These bacterial metabolites represent a link between the intestinal microbiota and the host organism and are an important component for the maintenance of homeostasis. Indeed, recent studies have described the beneficial effects of SCFAs in murine models of colitis and asthma through the regulation of immune cell

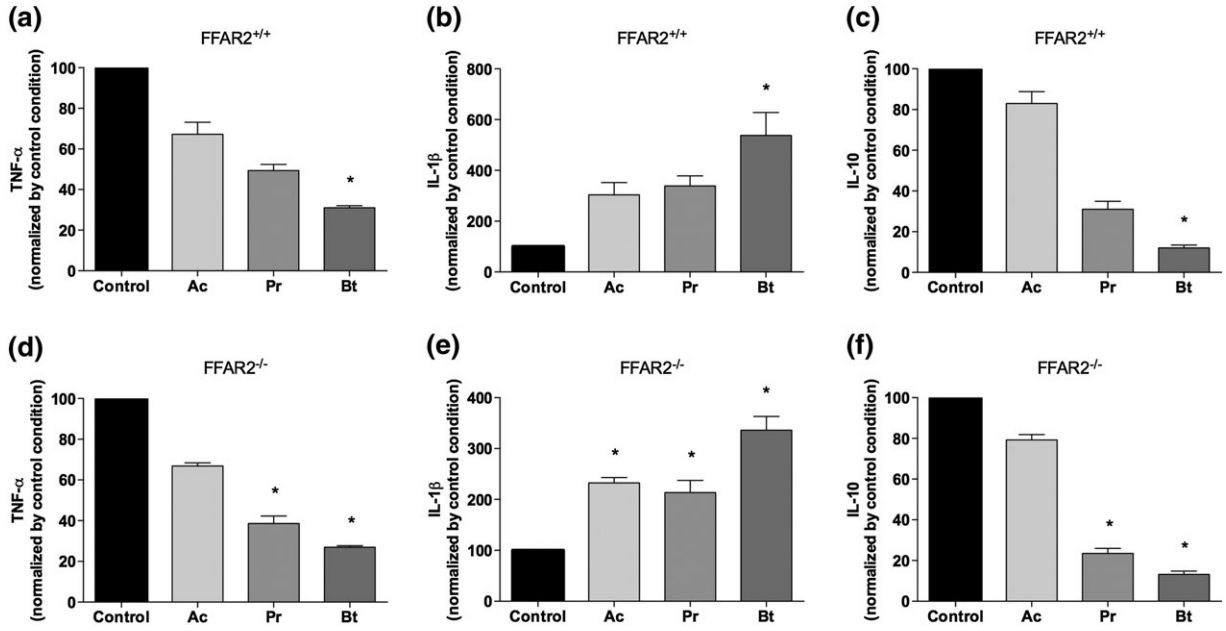

FIGURE 6 Short-chain fatty acid (SCFA) effects on cytokine production by neutrophils are FFAR2 independent. Neutrophils from FFAR2-deficient ($FFAR2^{-/-}$) and their controls ($FFAR2^{+/+}$) were incubated for 6 hr in the presence of SCFAs (control = phosphate-buffered saline, Ac = acetate 25 mM, Pr = propionate 8 mM, and Bt = butyrate 3.2 mM) and *Aggregatibacter actinomycetemcomitans* (multiplicity of infection 10:1). Tumour necrosis factor-α (TNF-α) (a and d), interleukin (IL) 1β (b and e), and IL-10 (c and f) concentrations were determined by ELISA. *N* = 10–12 animals for $FFAR2^{-/-}$ and 4–5 animals for $FFAR2^{+/+}$. All the results are presented as the mean ± SEM. $^{*}p < .05$ compared with the control condition

activation, including macrophages and dendritic cells, and the generation of T regulatory cells (Arpaia et al., 2013; Furusawa et al., 2013; Smith et al., 2013; Thorburn et al., 2015; Trompette et al., 2014).

Despite the vast literature focused on understanding the role of SCFAs in inflammatory conditions, a limited number of studies have investigated their role in infection, and particularly, in a key mechanism of defence, the neutrophil effector functions. It is worth mentioning that these bacterial metabolites are produced by bacteria commonly associated with infectious conditions, such as periodontopathogens *A. actinomycetemcomitans*, *Porphyromonas gingivalis*, and *Fusobacterium nucleatum* (Kurita-Ochiai, Ochiai, & Fukushima, 1998; Yu et al., 2014). Indeed, changes in their local tissue concentrations, which also reflect the metabolism of the dysbiotic microbiota, have been reported during diseases such as periodontitis and vaginosis (Al-Mushrif et al., 2000; Lu et al., 2014). In this context, some studies reported detrimental effects of SCFAs produced in periodontal tissue in nonimmune cells, as recently reviewed by Cueno and Ochiai (2016). Additionally, several anaerobic bacteria, which produce large amounts of SCFAs, have been recovered from abscesses in different tissues; treatment is normally very complicated in these conditions (Brook, 2016; Ladas, Arapakis, Malamou-Ladas, Palikaris, & Arseni, 1979).

Considering the scarcity of information, we proposed to analyse the effect of SCFAs in the context of an infection, particularly considering the effector mechanisms of neutrophils. For that, we employed a subcutaneous infection model with the opportunist pathogen *A. actinomycetemcomitans*, a facultative anaerobe Gram-negative bacterium associated with oral (periodontitis) and extraoral infections, including endocarditis and brain abscesses (Rahamat-Langendoen et al., 2011; van Winkelhoff & Slots, 1999). The subcutaneous chamber model used in this study was chosen because it provides an isolated environment that is permissive for the immune components and capable of holding a monoinfection, allowing for the investigation of the effects of SCFAs without the interference of other factors, such as tissue microbiota. In this sense, this is a very useful model for investigating host–pathogen interactions (Mydel et al., 2006; Ramsey, Rumbaugh, & Whiteley, 2011; Wang et al., 2014). In this study, we used the JP2 strain of *A. actinomycetemcomitans*, which is known to produce high amounts of leukotoxin that destroys human immune cells including neutrophils and is implicated in rapidly progressing forms of aggressive periodontitis (Haubek & Johansson, 2014). This strain induces periodontitis in mice after oral administration (Garlet et al., 2007; Repeke et al., 2010) and causes intense lesions after subcutaneous inoculation in mice (Ebersole, Kesavalu, Schneider, Machen, & Holt, 1995).

Short-chain fatty acids induce neutrophil chemotaxis under noninflammatory conditions (Le Poul et al., 2003; Maslowski et al., 2009; Sina et al., 2009; Vinolo et al., 2009; Vinolo, Ferguson et al., 2011). However, in the presence of chemokines or inflammation, this scenario is less clear (Rodrigues, Takeo Sato, Curi, & Vinolo, 2015). In the infection model used in this study, we found that the total number of cells within the chamber drastically increases over time, with a peak after 24 hr of the bacteria inoculation. However, no difference in total leukocyte or neutrophil migration was observed in the presence of the SCFAs. This absence of an effect may be in part explained by the fact that during an infection, SCFAs as well as other factors derived from the pathogen or the host cells (i.e., the ELR-CXCL chemokines, which act in neutrophil migration and include Cxcl1, Cxcl2, Cxcl5, and Cxcl7 in mice) form a complex mixture of chemoattractants that drives cells to the site of infection. In this context, SCFAs may have an irrelevant participation because neutrophils may prioritize end-target chemoattractants, such as fMLP, complement components, and others (Heit, Tavener, Raharjo, & Kubes, 2002). Additionally, SCFAs

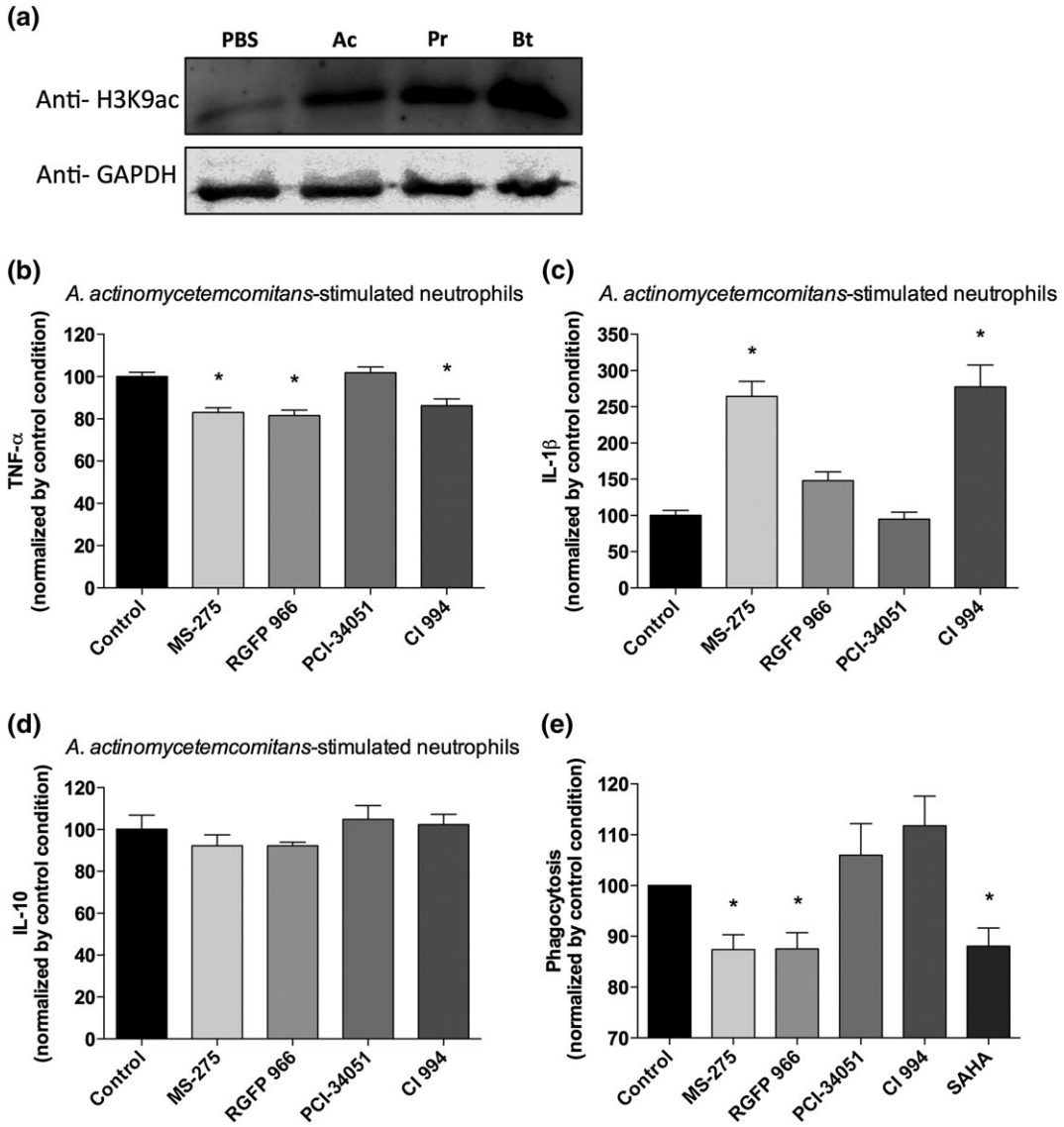

FIGURE 7 Modulation of neutrophil function by histone deacetylase inhibitors. Histone acetylation (H3K9ac) was measured in neutrophils incubated with short-chain fatty acids (Ac = acetate 25 mM, Pr = propionate 8 mM, and Bt = butyrate 3.2 mM) for 2 hr (a). Representative of three experiments. Tumour necrosis factor (TNF-α) (b), interleukin (IL) 1β (c), and IL-10 (d) were measured in the supernatants of neutrophils incubated for 6 hr in the presence of 10 μM HDACis and *Aggregatibacter actinomycetemcomitans*. Phagocytosis assays were performed with neutrophils incubated with pHrodo-marked bacteria (*A. actinomycetemcomitans*), previously opsonized, in the presence of 10 μM HDACis for 2 hr (e). *N* = 4–6 animals (b–d) and 11 animals (e). All the results were normalized by control values (C = 100%) and are presented as the mean ± SEM. $^{*}p < .05$ compared with the control condition

interference with chemokine production, as shown in this study for Cxcl2, which concentration was reduced in the presence of SCFAs, may also play a role in their final effect in this context.

Despite the same leukocyte migration to the chamber, we found that the number of viable bacteria was higher in the group of animals in which *A. actinomycetemcomitans* was inoculated with the SCFAs. The possibility that the presence of SCFAs affected the growth of the bacteria, as previously shown by Huang, Alimova, Myers, and Ebersole (2011), was excluded because no effect was observed in *A. actinomycetemcomitans* growth in the presence of neither isolated nor combined SCFAs in vitro, as shown here. A direct cytotoxic effect of the SCFAs on neutrophils, as previously described (Aoyama, Kotani, & Usami, 2010; Maslowski et al., 2009), was also absent. Taken together, these results indicated that SCFAs likely modulate effector mechanisms of neutrophils instead of inhibiting bacterial growth or inducing neutrophil death.

Phagocytosis is an essential effector mechanism of neutrophils for eliminating bacteria and other microorganisms. This process depends on the recognition of opsonins produced by the host, including complement, acute phase proteins, and antibodies by neutrophils receptors (e.g., Fc gamma receptor, CR1, and CR3; Nordenfelt & Tapper, 2011). This is the initial step for the activation of microbicidal mechanisms, including the generation of reactive oxygen species by the NADPH oxidase system and the release of several enzymes from granules, such as elastase, lysozyme, cathepsins, and defensins, which is also controlled by the parallel activation of other receptors, including the TLRs (Hayashi, Means, & Luster, 2003). In this study, we found that SCFAs impair the phagocytosis of bacteria both in vivo and in vitro at

concentrations found in infection sites. This effect was not associated with activation of FFAR2 but seems to involve the inhibition of HDACs. We found that SCFAs, at the concentrations used in the in vitro experiments, increased the acetylation of lysine 9 in histone 3 and that HDACis, including SAHA (a pan-HDACi), MS275 (a selective inhibitor of isoforms 1 and 3 of HDAC), and RGFP 966 (inhibitor of HDAC3), presented the same pattern of response compared to SCFAs. Roger et al. (2011) found that HDACis reduce the expression of phagocytic receptors in macrophages and their capacity to internalize and kill bacteria. Importantly, the authors of this study also demonstrated that HDACis, such as valproate, which acts in the same HDAC isoforms as the SCFAs, impair the innate defences of the host against microorganisms, leading to an increased susceptibility to bacterial and fungal infections. Moreover, a reduction in phagocytosis and the killing of other bacteria (*E. coli* and *Staphylococcus aureus*) were also observed in macrophages incubated with HDACi (Mombelli et al., 2011). In this context, our results extend the inhibitory effect of HDACi to other essential cells in the initial defence of the organism, the neutrophils. We also highlight the possibility that one specific isoform, HDAC 3, may have a prominent role in this effect of HDACi. However, it is worth mentioning that in the case of the SCFAs, HDAC inhibition is probably not the sole mechanism because acetate, the less potent inhibitor of HDAC among the three tested SCFAs, presented a similar impairment in phagocytosis as butyrate.

Neutrophils are also an important source of cytokines and chemokines in the acute response (they are the first and predominant leukocytes in the beginning of an immune response), which play a major role by orchestrating the progression of the process (Mantovani, Cassatella, Costantini, & Jaillon, 2011). Our in vivo experiments showed that 4 hr after inoculation of *A. actinomycetemcomitans* in the chamber, the levels of cytokines and chemokines rapidly increased (25–2,000 times). However, at this time point, no differences were observed between the groups with or without SCFAs. At 24 hr, the presence of SCFAs led to significant reductions in TNF-α, IL-10, and Cxcl2 concentrations in the chamber, an effect that was not observed for other cytokines. In vitro, the incubation of *A. actinomycetemcomitans*-stimulated neutrophils with the same mix of SCFAs used in the chamber resulted in reductions in the production of TNF-α (in both human and murine cells) and IL-10, even though there was an increase in the levels of IL-1β. Incubation of neutrophils with individual SCFAs and *A. actinomycetemcomitans* demonstrated that the most potent SCFAs regarding the effects on cytokines are butyrate and propionate. These data corroborate previous studies performed with rat and isolated human neutrophils stimulated with LPS (Tedelind, Westberg, Kjerrulf, & Vidal, 2007; Vinolo, Ferguson et al., 2011). The inhibition of TNF-α and IL-10 mRNA expression in response to *A. actinomycetemcomitans* by butyrate further supports the idea that the SCFAs interfere with intracellular pathways involved in the activation of gene expression, including transcription factors such as nuclear factor-κB (Vinolo, Ferguson et al., 2011; Machado et al., 2012) and, potentially, transcriptional repressors as observed in macrophages (Roger et al., 2011).

Contrary to other studies performed with primary epithelial cells or immortalized cell lines (Kim et al., 2013), the effect of SCFAs on cytokine production by neutrophils was found to be independent of FFAR2 activation. Again, we observed that some of the effects of SCFAs were mimicked by HDACis. The treatment of neutrophils with the HDACis MS-275 and CI994 and, to a lesser extent RGFP966, led to a similar response to *A. actinomycetemcomitans* regarding TNF-α and IL-1β production. However, for IL-10, the pattern of response observed with the HDACis was different from the SCFA results, indicating that for this cytokine, different HDACs may act together or that other mechanisms of regulation (including other isoforms of HDACs) are involved. In this sense, Villagra et al. (2009) demonstrated that HDAC11 is relevant for IL-10 production in response to TLR agonists in macrophages: Overexpression of this HDAC isoform suppressed IL-10 production, although its blockage had the opposite effect.

Interestingly, we observed that the SCFAs increase the production of IL-1β by neutrophils in vitro. This effect was not associated with an increase in this gene transcription, as shown in this study, or caspase activation (no significant effect of SCFAs was observed in vitro on caspase-1 activation in neutrophils, and the increase in IL-1β production was still present in cells treated with the pan-inhibitor of caspase Q-VD-OPh; data not shown). A recent paper showed that HDACis, including butyrate, promote the production and release of IL-1β through a caspase-1 independent mechanism in macrophages (Stammler et al., 2015). Our results suggest that this mechanism is also present in neutrophils, but its relevance in vivo is unclear because in our model, no difference in IL-1β was observed after infection.

Although several studies have been recently published showing the relevance of the metabolic and immunomodulatory effects of SCFAs, few studies have investigated their role in the context of host–pathogen interactions. SCFAs, produced by the pathological bacteria and/or the dysbiotic microbiota, can be found at high concentrations at sites of bacterial infections. For viral infections, there is some evidence that the presence of SCFAs facilitates viral reactivation through inhibition of class-1/2 HDACs of Epstein–Barr virus and latent HIV-1 (Imai, Yamada, Tamura, Ochiai, & Okamoto, 2012; Imai, Inoue et al., 2012). More recently, Yu et al. (2014) described that the saliva of patients with severe periodontal disease presents higher amounts of SCFAs when compared to healthy individuals and that these metabolites (mainly butyrate) also induce Kaposi's sarcoma-associated herpesvirus lytic gene expression and replication by the same mechanism. Nevertheless, regarding bacterial infections, especially extraintestinal infections caused by SCFA-producing microorganisms such as *A. actinomycetemcomitans*, the literature is limited.

Neutrophils present a prominent role in the periodontal pathogenesis; these cells, which are targets of the leukotoxin produced by *A. actinomycetemcomitans*, can eliminate these bacteria by phagocytosis and activation of both oxygen-dependent and independent mechanisms (Guentsch et al., 2009). This study showed that under infectious conditions caused by *A. actinomycetemcomitans*, the presence of SCFAs led to an attenuation of the neutrophil response against bacteria, mainly by reducing the production of inflammatory mediators and phagocytosis, thus facilitating the persistence of the microorganism. Additionally, our results suggest that the inhibition of specific isoforms of HDAC (1 and 3), but not activation of the FFAR2 receptor, the main SCFA-receptor expressed by neutrophils, accounts for the effects of SCFAs on neutrophils. Considering these findings and the literature, we hypothesize that in sites of anaerobe infection, including periodontal tissue and abscesses where the concentrations of the SCFAs in

direct contact with leukocytes and other cells are much higher than in the gut, instead of contributing to the maintenance of homeostasis, SCFAs exert a detrimental effect on the host by inhibiting not only neutrophils but also other immune cell functions favouring disease development and tissue destruction.

4 | EXPERIMENTAL PROCEDURES

4.1 | Animals

All procedures with animals were approved by the Ethics Committee on Animal Use of the Institute of Biology, University of Campinas (protocol numbers 3230–1 and 3667–1). Male C57BL/6 mice were provided by the Multidisciplinary Centre for Biological Investigation. FFAR2-deficient mice ($FFAR2^{-/-}$) were produced as previously described (Maslowski et al., 2009) and maintained in a C57BL/6 background in the animal facility of the Department of Genetics, Evolution and Bioagents of the Institute of Biology, University of Campinas. All mice were kept in regular filter-top cages and had free access to water and sterile food. The animals were used for the experiments at 8–10 weeks of age.

4.2 | Bacteria cultures

A. actinomycetemcomitans JP2 strain was provided by Dr. Mário Júlio Avila-Campos (Institute of Biomedical Sciences, University of São Paulo). Bacteria were cultivated in BHI chocolate agar supplemented with hemin (5 μg/ml) and menadione (1 μg/ml) at 37 °C in anaerobic conditions. For the experiments, isolated colonies of *A. actinomycetemcomitans* were collected in sterile PBS (pH = 7.4) after 48 hr of growth, centrifuged at 12,000 rpm for 5 min, and then washed twice with PBS. McFarland standards were used as a reference to adjust the densities of bacteria for the experiments. In all the experiments, the bacteria suspension was replated to confirm the absence of contamination.

4.3 | Mouse subcutaneous monoinfection model

The chamber implantation and the bacteria inoculation were performed according to the protocol proposed by Genco et al. (1991). Briefly, following anaesthesia, a trichotomy of the dorsal-lumbar region of the mice was performed. Skin disinfection was performed with 70% ethanol, and an incision of 1.5 cm was made in the region for subcutaneous implantation of a sterile coil-shaped stainless chamber. The incision was closed, and a degerming PVPI solution was applied. Animals were allowed to rest for 10 days, by which time the tissue was totally healed. No signs of infection were observed in the animals during the experimental protocol.

4.4 | Chamber inoculation with *A. actinomycetemcomitans*

Ten days after the chamber implantation, 100 μl of bacteria (1×10^8 CFUs) in PBS (with or without a mix of SCFAs) was inoculated inside the chamber using a hypodermic syringe with a 25-G sterile needle. The control groups contained PBS or PBS combined with a mix of SCFAs without the bacteria. After inoculation in the chamber, part of the bacteria suspension was plated in agar chocolate for counting of CFUs and discarding the presence of contamination. In these in vivo experiments, SCFAs were used in a mix containing 26 mM of acetate, 10 mM of propionate, and 2.5 mM of butyrate. The composition of this mix was based on a previous study (Lu et al., 2014), which described the concentrations of these bacterial metabolites in the gingival crevicular fluid of patients with aggressive generalized periodontitis before treatment.

4.5 | Chamber exudate analysis

At different time points after bacteria inoculation (4, 24, and 72 hr), the animals were euthanized by cervical dislocation, and the inflammatory exudate within the chamber was collected. To avoid coagulation of the collected material, we used hypodermic syringes filled with Ethylenediaminetetraacetic acid (EDTA) (10 μl of a 10% EDTA solution). A minimum of 90 μl of chamber fluid was collected from each mouse and separated for different analyses. Part of the exudate (20 μl) was used for quantification of TNF-α, IL-1β, IL-6, IL-10, IL-12, Cxcl1, and Cxcl2 by ELISA following the instructions of the company (R&D Systems, Minneapolis, MN, USA). The total number of cells was determined using a Neubauer chamber. Additionally, cytocentrifuge preparations of the chamber exudate were stained and used to evaluate the leukocytes in the fluid (neutrophils and mononuclear cells).

Part of the exudate (10 μl) was diluted in sterile saline and plated in BHI chocolate agar. The plates were maintained at 37 °C for 3–5 days in anaerobic conditions and then used for counting CFUs. On the basis of this number, which refers to the amount of bacteria that survived within the chamber, and considering the total number of CFUs of the inoculum, we determined the viability of the bacteria in the chamber.

4.6 | Evaluation of phagocytosis in vivo

A suspension of bacteria was suspended in PBS (pH = 9.0) and marked with the pHrodo dye (Invitrogen) while stirring at 37 °C for 30 min. Next, they were washed in PBS (pH = 7.3) and resuspended in PBS with or without SCFAs (at the same concentrations used before). Next, the marked bacteria were inoculated within the chambers in the mice at a concentration of 1×10^8 CFUs. Four hours later, the animals were euthanized, and the inflammatory exudate was collected. After that, the cells were marked using anti-Ly6G (APC antimouse Ly-6G Clone 1A8, BioLegend) for at least 15 min and analysed by flow cytometry (BD FACSCalibur). For this experiment, trypan blue was used for quenching the fluorescence of the externally bound bacteria.

4.7 | Growth curve of *A. actinomycetemcomitans*

Short-chain fatty acids, combined in a mix or isolated, were tested to check whether they interfered with *A. actinomycetemcomitans* growth in vitro. The bacteria density was adjusted to 1.5×10^8 CFU/ml, and they were incubated in the presence of the indicated concentrations of SCFAs at 37 °C. The optical density at 600 nm was determined after

0, 2, 4, 6, 12, 24, and 48 hr of incubation. The 24-hr samples were serially diluted and plated on BHI chocolate agar for CFU determination.

4.8 | Experiments with isolated neutrophils

For the in vitro experiments, elicited neutrophils were obtained after administration of a sterile solution of 4% thioglycollate in the intraperitoneal region. Animals were euthanized after 4 hr, and cells were collected with an intraperitoneal wash using saline solution. These cells are previously activated; therefore, they show similar functional characteristics to what would be expected during an immune response (Itou, Collins, Thoren, Dahlgren, & Karlsson, 2006). Nontoxic concentrations of SCFAs, as determined by an MTT test, were used in the experiments.

Tumour necrosis factor-α, IL-1β, IL-10, and Cxcl2 concentrations in the culture supernatant were evaluated by ELISA (Duo Set Kit, R&D System, Minneapolis, MN, USA). Briefly, neutrophils were plated at a concentration of 1×10^6 cells/ml at 37 °C in a 5% CO_2 atmosphere and maintained in RPMI 1640 medium without antibiotics and with 10% inactivated fetal bovine serum. Cells were incubated for 6 hr after being stimulated with LPS (2.5 μg/ml) or with bacteria at a proportion of 10:1 (bacteria:neutrophils). Tests were performed with cells treated with acetate, butyrate, and propionate mixed or individually at different concentrations, as indicated in the results. For the experiments with HDAC inhibitors (HDACi), we used drugs capable of inhibiting different HDAC isoforms: MS-275 for the isoforms 1 and 3, RGFP 966 for the isoform 3, PCI-34051 for the isoform 8, and CI 994 for the isoform 1. These inhibitors were all dissolved in DMSO and used in nontoxic concentrations (1 and 10 μM). For the controls, we added DMSO diluted in culture medium at the highest concentration (0.1%).

4.9 | In vitro phagocytosis assay

The phagocytosis assays were performed using a ratio of 1:50 (neutrophils:bacteria). Neutrophils (obtained as described above) were incubated with the treatment and the bacteria previously opsonized with murine serum and labelled with pHrodo dye (Invitrogen). Samples were incubated in RPMI 1640 medium without antibiotics containing 10% of inactivated fetal bovine serum and were stirred for 120 min at 400 rpm and 37 °C. After the incubation period, samples were washed, resuspended in 100 μl PBS, and marked with anti-Ly6G (PE antimouse Ly-6G Clone 1A8, BioLegend). Next, trypan blue was added to quench the fluorescence of the noninternalized bacteria (Simons, 2010). Negative controls consisting of cells incubated alone or for a very short time (2 min) were also evaluated. The samples (10,000 events) were analysed by flow cytometry (BD FACSCalibur).

4.10 | Experiments with human peripheral blood neutrophils

Human neutrophils were isolated from the blood of healthy volunteers by using Histopaque 1077 (Sigma-Aldrich, St. Louis, MO, USA) density gradient separation. Erythrocytes were removed by using a hypotonic lysis buffer, and the cells were then tested for cytokine production and phagocytosis of *A. actinomycetemcomitans* in the presence of different dilutions of an SCFA mixture. Human neutrophils were incubated for 6 hr in the presence of bacteria and SCFAs before the supernatant was collected for TNF-α measurement. For the phagocytosis assay, we followed the same protocol previously described with the exception of the antibody used for neutrophils identification, which in this case was the antihuman CD15 APC-conjugated (clone HI98 from Immunotools). Ethical approval was provided by the Ethics Committee in Research of the University of Campinas, CAAE: 002/201160895716.7.0000.5404.

4.11 | Statistical analysis

All the analyses were performed using GraphPad software 5.0 (Graph Pad Software, Inc., San Diego, CA, USA), and the differences were considered significant for $p < .05$. The results were analysed using D'Agostino–Shapiro–Wilk normality tests. Differences between two groups were compared by Student's *t* tests or Mann–Whitney test for parametric or nonparametric data. For more than two groups, the differences were compared by one-way analysis of variance followed by Tukey's post hoc test.

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CONFLICT OF INTEREST

The authors have no conflict of interest.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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