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# Butyric acid stimulates bovine neutrophil functions and potentiates the effect of platelet activating factor

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ABSTRACT

Increased short-chain fatty acid (SCFA) production is associated with subacute ruminal acidosis (SARA) and activation of inflammatory processes. In humans and rodents, SCFAs modulate inflammatory responses in the gut via free fatty acid receptor 2 (FFA2). In bovines, butyric acid is one of the most potent FFA2 agonists. Its expression in bovine neutrophils has recently been demonstrated, suggesting a role in innate immune response in cattle. This study aimed to evaluate if butyric acid modulates oxidative and non-oxidative functions or if it can potentiate other inflammatory mediators in bovine neutrophils. Our results showed that butyric acid can activate bovine neutrophils, inducing calcium ($Ca^{2+}$) influx and mitogen-activated protein kinase (MAPK) phosphorylation, two second messengers involved in FFA2 activation. $Ca^{2+}$ influx induced by butyric acid was dependent on the extracellular and intracellular $Ca^{2+}$ source and phospholipase C (PLC) activation. Butyric acid alone had no significant effect on reactive oxygen species (ROS) production and chemotaxis; however, a priming effect on platelet-activating factor (PAF), a potent inflammatory mediator, was observed. Butyric acid increased CD63 expression and induced the release of neutrophil granule markers matrix metalloproteinase-9 (MMP-9) and lactoferrin. Finally, we observed that butyric acid induced neutrophil extracellular trap (NET) formation without affecting cellular viability. These findings suggest that butyric acid, a component of the ruminal fermentative process, can modulate the innate immune response of ruminants.



## 1. Introduction

SARA is a common digestive disorder prevalent in high-producing dairy herds (Kleen et al., 2003). This disorder often precedes excessive ingestion of non-structural carbohydrates that leads to the production and accumulation of SCFAs within the rumen (Plaizier et al., 2008). The principal SCFAs are acetic acid (C2), propionic acid (C3) and butyric acid (C4), which constitute 95% of all SCFAs (Bergman, 1990). Rumenitis is a frequent sequel to SARA, characterised by accumulation of neutrophils in the ruminal epithelium (Enemark, 2008; Thomson, 1967); however, the pathogenesis of this condition is not fully understood. Some studies suggest that increased SCFA production, particularly of butyric acid and propionic acid, may be involved (Enemark, 2008; Krehbiel et al., 1995). SCFAs are relevant modulators of the inflammatory response, as they are capable of activating neutrophils (Vinolo et al., 2011b). Neutrophil recruitment and activation are key events in the inflammatory response and are critical for host defence against invading microbial pathogens in cattle (Burvenich et al., 1994; Paape et al., 2003). Neutrophils can eliminate pathogens at the site of infection via three different microbicidal mechanisms: the oxidative mechanism through the generation of ROS, the non-oxidative mechanism by releasing degradative enzymes stored in cytoplasmic granules and NET formation (Kolaczkowska and Kubes, 2013). However, these mechanisms can also induce tissue damage as part of the inflammatory process (Nathan, 2006). Neutrophils migrate towards a gradient of chemical messengers (chemoattractants) at sites of infection and inflammation, produced by host cells and microorganisms (Wang, 2009). PAF is a biologically active phospholipid that acts as a potent chemoattractant and inflammatory mediator for bovine neutrophils. In these cells, PAF can induce $Ca^{2+}$ mobilisation, MAPK phosphorylation, granule release and ROS production (Hidalgo et al., 2004; McClenahan et al., 2000; Sandoval

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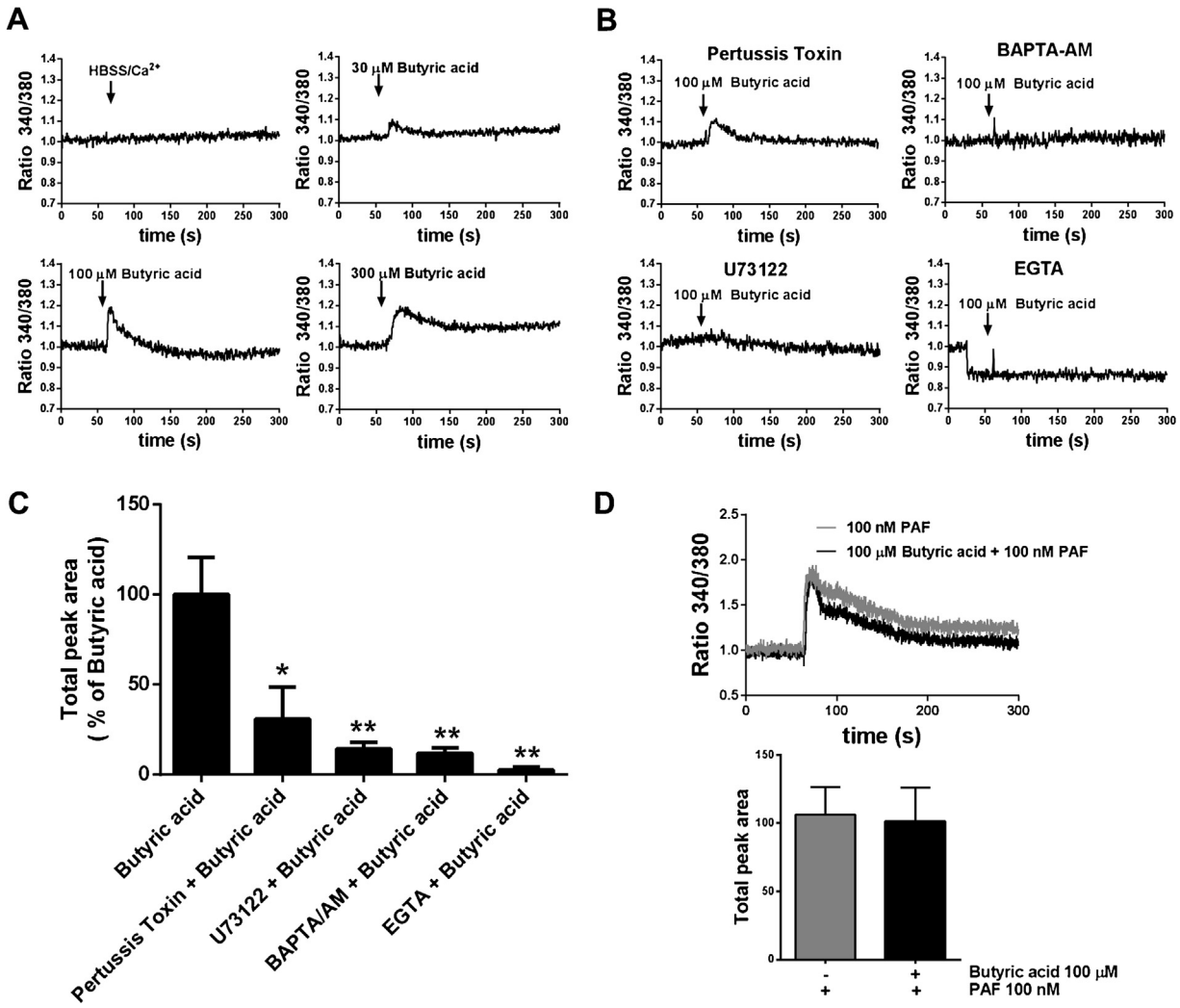


**Fig. 1.** Butyric acid induces $Ca^{2+}$ influx in bovine neutrophils. (A) Fura 2-AM-loaded cells were treated with 30, 100 and 300 μM butyric acid or vehicle (HBBS/$Ca^{2+}$), and $Ca^{2+}$ influx was measured as the increase in the fluorescence ratio 340/380. Arrows indicate the time of application of butyric acid or vehicle and the records are representative of independent experiments. (B) The neutrophils were incubated for 1 h with pertussis toxin, 5 min with 2 μM U73122, 30 min with 50 μM BAPTA-AM and 30 s with 0.3 mM EGTA before stimulation with 100 μM butyric acid. Arrows indicate the time of application of butyric acid, and the records are representative of independent experiments. (C) Total peak area of experiments in (B) was calculated and expressed as percentage of butyric acid. Each bar represents the mean ± SEM of four experiments, $^{*}P < 0.05$ and $^{**}P < 0.01$, compared with butyric acid treatment. (D) The effect of butyric acid on $Ca^{2+}$ influx induced by 100 nM PAF. Neutrophils were incubated for 10 min with 100 μM butyric acid before stimulation with PAF. Total peak area was calculated and each bar represents the mean ± SEM of three experiments.

et al., 2007b; Swain et al., 1998) via a G protein-coupled receptor (GPCR) (Burgos et al., 2004).

SCFAs show anti- and pro-inflammatory properties. In this sense, butyric acid decreases several neutrophil functions, such as phagocytosis and killing activity, via reduced ROS production (Vinolo et al., 2009a). Studies performed in human and mouse neutrophils demonstrated that SCFAs have a significant chemotactic effect (Le Poul et al., 2003; Sina et al., 2009; Vinolo et al., 2009b) and increase expression of the L-selectin adhesion molecule (Vinolo et al., 2009b).

SCFA-induced effects are attributed to their ability to activate two GPCR, FFA2 and FFA3. GPCR stimulation triggers neutrophil activation, which produces an increase in intracellular $Ca^{2+}$ levels and activation of extracellular-signal-regulated kinase 1/2 (ERK1/2) and p38 MAPK signalling pathways (Lacy and Eitzen, 2008). FFA2 and FFA3 are expressed in human and bovine neutrophils, and the expression of FFA2 is considerably higher (Carretta et al., 2013; Le Poul et al., 2003). SCFAs are primarily natural agonists of FFA2 and have species-dependent variations in the ligand response (Hudson et al., 2012), indicating a general species adaptation mechanism according to its metabolism and bacterial environment (Gloriam et al., 2007). FFA receptors are activated by SCFAs with a different order of potency, according to chain length. For the human FFA2 receptor, the order of potency is C3 > C2 = C4 (Le Poul et al., 2003), while the bovine receptor order of potency is C4 > C3 > C2 (Hudson et al., 2012). In fact, SCFA potency increases with longer chain lengths in the bovine receptor, suggesting that this receptor may serve different functions in cattle compared with humans.

We hypothesised that butyric acid can activate oxidative and non-oxidative responses and potentiate the effects induced by other mediators such as PAF in bovine neutrophils.

## 2. Materials and methods

### 2.1. Animals, blood collection and neutrophil isolation

Five clinically healthy, non-pregnant black Friesian dairy heifers were used in all of the experiments. The animals were maintained at the Universidad Austral de Chile. Blood was collected by jugular venepuncture directly into acid citrate dextrose (ACD) Vacutainer