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Abstract

Short chain fatty acids (SCFAs) have been recognized as mediators of immune responses, including pathways of cytokine production. In this study, we investigated the immune-regulatory effects of SCFAs on human peripheral blood mononuclear cells (PBMCs) from buffy coat of healthy donors. PBMCs were exposed to varying concentrations of individual SCFAs or their mixtures, and, the production of interleukin (IL) IL-1β, IL-2, IL-6, IL-10, IL-17 IL-21 IL-23 and transforming growth factor beta 1 (TGF-β1) were assessed. T cell differentiation after exposure to SCFAs was also examined. In comparison to LPS-stimulated cells (controls), SCFAs slightly decreased the production of TGF-β1 and significantly reduced IL-6 production ($p < 0.05$) and butyrate was more effective than acetate or propionate; non-stimulated cells did not respond to SCFAs. In addition, the viability of PBMCs was not significantly affected. SCFAs particularly butyrate caused the induction of $CD4^+CD25^+$ regulatory T cells (Treg) rather than Th17 cells. It is clear that. SCFAs may up-regulate the production of anti-inflammatory cytokines in PBMCs resulting in the induction of $CD4^+CD25^+$ Treg cells.

Key words: Short chain fatty acids, Human peripheral blood mononuclear cells, Cytokines, Immune modulation, T helper cells, Th17 and $CD4^+CD25^+$ Treg cells.

1. Introduction

Short chain fatty acids (SCFAs) are a sub-group of fatty acids with 2 to 6 carbon atoms (C_2 - C_6 mono-carboxylic acids), formed principally from fermentation of prebiotics by anaerobic micro-organisms in the colon (Cummings & Macfarlane, 1991). SCFAs are predominant anions in human colon contents (Sellin, 1999). Relative proportions and amounts of SCFAs vary according to the type of fibre in the diet, type and population of microflora present in the colon and the gut transit time, absorption and utilization by colonic epithelium (Macfarlane & Macfarlane, 2003). Hence, the concentrations of SCFAs in the colon, portal blood and peripheral circulation are different. Total SCFAs in the colon has been estimated to range from 70 - 140 mM with concentration ratios of acetate, propionate and butyrate being, 10, 1.5 and 2 mM, respectively (Bergman, 1990; Cummings, Pomare, Branch, Naylor, & Macfarlane, 1987; Sellin, 1999; Topping & Clifton, 2001). Whereas in the portal blood, concentrations are typically higher (0.375 mM) compared to that in peripheral circulation (0.079 mM) (Cummings et al., 1987). Likewise, Meijer, de Vos, and Priebe (2010) reported the total concentration of SCFAs in human peripheral blood to ranged from 0.050 – 0.1 mM and in portal blood, about 0.3 – 0.450 mM.

SCFAs have been shown to have beneficial effect on human health; they are the favoured fuel of the colonic epithelium and are vital for intestinal and epithelial barrier functions (Adom & Nie, 2013; Scheppach, 1994). Lack of source of energy may lead to diminished integrity of epithelial function resulting in bowel disorders such as bowel inflammation (Harig, Soergel, Komorowski, & Wood, 1989; Wong & Jenkins, 2007). Furthermore, SCFAs stimulate colonic blood flow and enhance fluid and electrolytes uptake such as calcium absorption (Roy, Kien, Bouthillier, & Levy, 2006). Recent interest in SCFAs benefits have focused on their regulatory effects on immune responses by affecting the immune cells functions (Vinolo, Rodrigues, Nachbar, & Curi, 2011). SCFAs may ameliorate some

pathological conditions such as inflammatory bowel disease (IBD), possibly via their effect on immune responses (Tedelind, Westberg, Kjerrulf, & Vidal, 2007; Vernia et al., 1995; Vinolo et al., 2011). However, the mechanism of action is not clearly demonstrated but it has been reported that SCFAs may trigger cellular receptors such as G protein coupled receptors (GPCRs), (GPR41 and GPR43), of immune cells and subsequently initiate the immune response (Bindels, Dewulf, & Delzenne, 2013; Brown et al., 2003; Le Poul et al., 2003; Maslowski et al., 2009; Masui et al., 2013). Moreover, studies have suggested that butyrate and other SCFAs have inhibitory effects on the nuclear factor kappa B (NF-κB) signalling (Liu et al., 2012; Luhrs et al., 2001; Segain et al., 2000; Tedelind et al., 2007) and histone deacetylase (HDAC) (Aoyama, Kotani, & Usami, 2010; Waldecker, Kautenburger, Daumann, Busch, & Schrenk, 2008). SCFAs may regulate cytokine production (Cavaglieri et al., 2003; Masui et al., 2013; Yin, Laevsky, & Giardina, 2001), and, recently, butyrate and propionate have been noted to promote peripheral regulatory T cell (Treg)differentiation which might contribute to immune homeostasis (Arpaia et al., 2013).

PBMCs compose mainly of lymphocytes, macrophages and monocytes. The lymphocyte population in healthy human adults consists of approximately 60% T cells ($CD4^+$ and $CD8^+$) and 35% B cells, natural killer cells (NK), macrophage and monocytes (Minoprio, 2000; Plebanski, 2002). PBMCs have been widely used in immunological and pharmaceutical studies probably due to their properties, which relate to sophisticated immune cells. The cells are co-cultured with different types of immune suppressant or stimulant drugs to study and determine their efficiencies using different parameters of immune responses such as the release of immune mediators (cytokines) (Ramachandran et al., 2012). T lymphocytes can be functionally distinguished into cytotoxic (Tc) and helper (Th) cells. Human naive $CD4^+$ T helper cells can be divided into different functional subsets Th1, Th2, Th17, and Treg cells according to surface phenotype and cytokine secretion (Luckheeram, Zhou, Verma, & Xia,

2012). Th1 cells express T-box transcription factor (Tbet-2) and release IL2, TNFα and IFNγ cytokines and act against intracellular pathogens. Th2 express GATA3, produce anti-inflammatory cytokines such as IL-1 receptor antagonist (IL-1ra), IL-4, IL-5, IL-6, IL-10 and IL-13, and stimulate humoral immune responses against extracellular pathogens. Th17 cells express the up-regulation of transcription factors, such as RORγt and STAT-3, release pro-inflammatory cytokines IL-17 and play a role in organ-specific autoimmune diseases (Thomas Korn, Oukka, Kuchroo, & Bettelli, 2007). Treg cells express FoxP3 and produce IL-10 and TGF-β cytokines, promote tolerance to self and non-pathogenic antigens, suppress amplitude immune and inflammatory responses, drive and modulate immune responses, and abrogate autoimmune diseases (Wan & Flavell, 2009).

Mature $CD4^+$ Th cells are also involved in autoimmunity, during T cell receptor (TCR) activation in a particular cytokine milieu, naive CD4 T cells may differentiate into one of several lineages of Th cells, including Th1, Th2, Th17, and induce Treg cells (iTreg) as defined by their pattern of cytokine production and function. Imbalance of either pattern of Th1 or Th2 cells may cause autoimmune disease e.g. a dysregulation of T helper cell phenotype in favour of Th1 appears to underlie Crohn's disease, a form of IBD (Dionne, Ruemmele, & Seidman, 2004). Along with Th1 and Th2 cells, (FoxP3) Treg and Th17 cells have been recognized as significant players in immune balance (Brand, 2009). The effect of SCFAs on Treg cells may subsequently regulate the induction of Th1, Th2 and Th17 and maintaining the immune homeostasis, thus SCFAs may be valuable to maintain the immune homeostasis and prevent of chronic inflammation (Bailon et al., 2010; Vinolo et al., 2011).

SCFAs particularly butyrate have been known to modulate immune responses (Cox et al., 2009; Vinolo et al., 2011). However, little is known about the effects of SCFAs on human PBMCs. Herein we evaluated the regulatory effects of SCFAs in PBMCs and the induction of Treg and/or Th17 cells. The regulatory effect of SCFAs in the production of pro- and anti-

inflammatory cytokines with PBMCs in the presence or absence of LPS stimulation was also examined.

2. Material and methods

2.1. Chemicals and reagent

Biological grade acetate, propionate, butyrate, purified LPS from *Escherichia coli* O111:B4 and growth medium Roswell Park Memorial Institute medium (RPMI-1640) were purchased from Sigma (Sigma-Aldrich, Sydney, Australia). Ficoll-Paque™ Plus was from GE Healthcare (GE Healthcare, Bio-Sciences, Uppsala, Sweden). Antibiotic-Antimycotic solution and Fetal bovine serum (FBS) were acquired from Gibco Life Technologies (Gibco®Life Technologies, Mulgrave, Australia). Phosphate-Buffered Saline (1X) pH 7.4, (PBS) was from Invitrogen (Invitrogen Pty Ltd., Mount Waverly, Victoria, Australia),The Buffy coat was provided by the Australian Red Cross Blood Services, Melbourne, Australia, Other reagents and chemicals were of biotechnological and molecular-biology grade from Sigma-Aldrich unless otherwise stated.

2.2. Isolation of human PBMCs from buffy coat using Ficoll gradient

In order to meet the requirements of the National Health and Medical Research Council "National Statement on Ethical Conduct in Human Research" (National Health and Medical Research Council, 2007), the Human research ethics of the proposed project was accepted and approved by the Chair of the Faculty of Health, Engineering, and Science, Victoria University Human Research Ethics Committee. A contract agreement was also conducted with Australian Red Cross Blood Services Melbourne Australia, in order to the supply of buffy coat.

PBMCs were isolated from human buffy coat by Ficoll-Paque gradient according to the method described by (Donkor et al., 2012) with minor modifications. Briefly, individual buffy coat (60 mL) was diluted with an equal volume of phosphate buffer saline (PBS) and layered on Ficoll-Paque Plus (GE Healthcare, Bio-Sciences, Uppsala, Sweden). Cells at the interphase were collected following centrifugation (680 *g*, 25 min, 18°C) (Sorvall® RT7 centrifuge; DuPont, Newtown, CT, USA). Separated layers PBMC were washed twice in cold PBS, and following centrifugation (680 *g*, 10 min, 18°C). To **lyse** any remaining red blood cells, the pellet was resuspended in 5 mL red blood cell lysing buffer; Ammonium-Chloride-Potassium (ACK) (Gibco® ACK Life Technologies) and incubated for 8 min at room temperature. The volume was then adjusted to 35 mL using sterile PBS then centrifuged (680 *g*, 10 min, 18°C). Following two subsequent washes, the cell pellet was resuspended in RPMI1640 medium supplemented with 10% FBS and 1% of Antibiotic-Antimycotic solution for co-culture and stimulation.

2.3. Co-culture and stimulation of PBMCs by SCFAs

Human PBMCs were seeded in flat bottom 6-well tissue culture plates (Corning, Sigma) at final concentration 1×10^6 cells/mL RPMI1640 medium per well, either in RPMI1640 medium alone or with LPS (controls), medium with LPS and acetate, propionate, butyrate or mixed SCFAs. The concentrations of SCFAs were 1, 1.5, 2 mM similar to physiological concentration found in the colon and peripheral circulation (portal vein) (Cummings et al., 1987; Topping & Clifton, 2001), similar concentrations were also used in previous studies (Liu et al., 2012; Nancey et al., 2002; Weber & Kerr, 2006). LPS (5 μg/mL) was used for cells stimulation (Chen, Bruns, Donnelly, & Wunderink, 2010; Jansky, Reymanova, & Kopecky, 2003). The plates were incubated at 37°C in a 5% CO_2 incubator for 48 hours.

To investigate the responses of PBMCs to the stimuli (LPS and/or SCFAs), the cells were stimulated with 5µg/mL LPS alone (Chen et al., 2010), with SCFAs only or a combination of LPS and SCFAs for 48 h. PBMCs were also stimulated with LPS for 24 hour then SCFAs were added and cells further incubated for another 24 hours.

2.4. PBMCs viability assay

PBMCs viability in presence of LPS and/or SCFAs were assessed using MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2- (4-sulfophenyl)-2H-tetrazolium, inner salt) assay (CellTiter 96® AQueous One Solution Cell Proliferation Assay, Promega, USA) according to manufacture instruction. Briefly, cells were seeded in 96-well plates (Corning, Sigma) at concentration 1×10^3 cells per 100 µL of growth media with or without various stimuli (as above), and then incubated for 48 h at 37°C in a 5% CO_2 incubator. The viability was detected by adding 20 µL of MTS solution, followed by 4 h of incubation then absorbance was read at 490 nm using iMark Microplate Absorbance Reader (BIO-RAD, Australia). After subtracting of the background reading, cell viability was calculated as:

% viable cell = (Optical density **(**OD) of SCFAs treated sample / OD of control sample) x 100.

2.5. **ELISA analysis of cytokines**

Supernatants from stimulated and non-stimulated PBMCs cultures were collected and analysed for cytokines concentrations using BD OptEIA ELISA kits (BD Bioscience, San Diego, CA), including IL-1β, IL-2, IL-6, IL-10, IL-17, IL-21 TGF-β1 and IL-23. The detection procedures were performed in accordance with the manufacturer's instructions. Cytokines were measured at 24 and 48 hours of stimulation. Data are expressed as the mean

cytokine response minus background (pg/ml) of each treatment from triplicate wells, plus or minus the standard error of the mean.

2.6. Measurement of nitric oxide production

Nitric oxide (NO) production from stimulated PBMCs and non-stimulated PBMCs was measured spectrophotometrically using Griess Reaction Assay (Promiga, Auburn, Australia) according to the manufacture's instruction. Briefly, after co-culture and stimulation of PBMCs (as above), 50 µL aliquots of supernatant were incubated with 100 µL Griess reagent (50 µL of 1% sulfanilamide in 0.1 M HCl and 50 µL of 0.1% N-1-napthylethylenediamine dihydrochloride) for 10 min at room temperature. Absorbance was read at 550 nm using iMark Microplate Absorbance Reader (BIO-RAD, Australia) and results were calculated based on a $NaNO_2$ standard curve.

2.7. Flow Cytometry Analysis of Th17 and Treg cells

Activated PBMCs were collected and analysed for induction of Treg/Th17 cells. Briefly, 1 mL suspension PBMCs culture was collected and centrifuged at 500*g* for 10 min, cell pellet was washed twice using fluorescence activated cell sorting (FACS) buffer (PBS + 2% FBS) and the suspension was centrifuged again at 500*g* for 10 min. PBMCs were re-suspended at 1 $\times 10^6$ cells/ml in FACS buffer and surface marker staining was performed using fluorescein isothiocynate (FITC)-labelled anti-human CD4, allophycocyanin labelled anti-human CD25/CD3 (Becton-Dickinson), peridinin chlorophyll protein (PerCP)-labelled anti-human CD3 (Biolegend, San Diego, CA, USA) and PerCP cyanine (Cy)5.5-labelled anti-human CCR6 (CD196). Intracellular staining was performed using phycoerythrin (PE)-labelled anti-human FoxP3/RORγt (BD Pharmingen and R&D Systems, Minneapolis, MN, USA, respectively), according to the manufacturer's instructions. Samples were read using a BD FACSCalibur and data was acquired using CellQuest program (Becton Dickinson

198 Biosciences). Analysis was performed using Gatelogic version 3.07 software (Inivai,
199 Victoria, Australia). Absolute numbers of Treg and Th17 cells were calculated as a
200 percentage of the total lymphocyte number (Donkor et al., 2012).

201 **2.8. Statistical analysis**

202 The results obtained were analysed as a split plot in time design with three main factors:
203 SCFAs (Three fatty acids) and their doses (3 dose levels) as the main plot and time (two time
204 frame 24 and 48) LPS was used as stimulant. The statistical evaluations of the data were
205 performed using the general linear model (GLM) (SAS/STAT., 1996). Significant differences
206 between treatments were tested by analysis of variance (ANOVA) followed by a comparison
207 between treatments performed by Fisher's least significant difference (LSD) between each
208 sample with a level of significance of ($p < 0.05$). Data are expressed as mean and standard
209 deviation of triplicate measures determined in 5 independent experiments.

210 **3. Results**

211 **3.1. PBMCs viability, proliferation and NO production**

212 The MTS assay of PBMC viability and proliferation with or without different stimuli is
213 shown in Figure 1. Proliferation of PBMC were potentiated in the presence of LPS in all
214 samples compared with non LPS-stimulated PBMCs. Relatively similar effects resulted in the
215 presence of each SCFA with maximum proliferation observed in the presence of butyrate
216 followed by mixed SCFAs over 48 h.Similar LPS effect on PBMCs proliferation was
217 reported (Jansky et al., 2003), and SCFAs did not induce cell apoptosis at used concentration.

218 Production of NO was enhanced in the presence of LPS, whereas the addition of SCFAs
219 induced remarkable reduction ($p < 0.05$) of NO (Fig. 2). After 48 h of incubation, NO was
220 reduced by 38.3% in the presence of butyrate and 29.9% in acetate and propionate compared

with LPS-stimulated cells, However the inhibition of NO in the SCFA mixture was similar to that of butyrate.

3.2. Production of cytokines

Figure 3 (A-H) show cytokines (IL-β1, IL-2, IL-6, IL-17, IL-21 IL-23 and TGF-β1) produced by PBMCs in the presence of LPS compared with non-stimulated PBMCs, the combination of LPS and SCFAs or SCFAs only. Results show significant ($p < 0.05$) decrease in IL-β1, IL-2, IL-6, IL-17 and IL-21, with a slight reduction of IL-23 and TGF-β1 in the presence of SCFAs, compared to cells stimulated with LPS only. However, IL-10 secretion was not affected by LPS compared to non LPS-stimulated cells but it was increased in the presence of SCFAs particularly in butyrate (Säemann et al., 2000). We have also noted similar results when PBMCs stimulated with LPS for 24 h followed by addition of SCFAs and incubated for another 24 hours (Table 1).

3.3. $CD4^+$ CD25; Th17 and Treg populations

Flow cytometric analysis of LPS and SCFAs stimulated PBMCs showed increased $CD4^+$ $CD25^+$ Treg and Th17 cells compared to unstimulated cells (Fig. 4). LPS aloneincreased the Th17 cell population compared to Treg cells after 48 h of incubation. In the presence of SCFAs particularly butyrate, the relative proportion of Th17 and Treg showed slight increase of Treg cells compared to Th17 (Fig. 4). The addition of SCFAs along with LPS at 0 time or after the pre-stimulated PBMCs with LPS, showed comparatively similar up-regulation of Treg cells after 24 h compared to unstimulated cells (control). Butyrate was more effective in induction of Treg cells than acetate or propionate. The addition of mixed SCFAs did not induce augmentation of the effects as compared with each SCFA. However, their effects were concentration-dependent; 2 mM of SCFAs was more effective in up-regulation of Treg than 1 and 1.5 mM, (Fig. 4).

4. Discussion

Despite most of intestinal SCFAs being used by intestinal epithelium cells, a considerable amount of acetate, propionate, and butyrate are absorbed into the blood and exert their effects at peripheral tissue level beyond the digestive system through regulation of immune responses (Matsumoto et al., 2006). Consequently, we assumed that SCFAs might have regulatory effects on peripheral PBMCs. Therefore, in the current study, we used *in vitro* LPS-stimulated PBMCs of healthy donor to compare the anti-inflammatory effect of acetate, propionate, and butyrate and regulation of Th17 and Treg balance. It was found that SCFAs reduced the production of pro-inflammatory factors including IL-1β, IL-6, IL-17 and NO whereas they enhanced the production of anti-inflammatory mediators such as IL-10, and IL-2. Furthermore, SCFAs affected gene expression of T helper cells possibly through their effect on immune mediators and growth factors such as IL-6 and TGF-β1, which might have led to the up-regulation of Treg cells. Interestingly, SCFAs exhibit these effects mainly in LPS-stimulated PBMCs whereas non LPS-stimulated cells were not affected by SCFAs.

4.1. Effect of SCFAs on cells viability

To determine whether SCFAs exert their effects through stimulation of PBMCs but not via induce cellular death, MTS assay was conducted after co-culturing of PBMCs with LPS and SCFAs (Fig. 1). Cell growth was slightly potentiated in the presence of LPS and SCFAs particularly butyrate or mixed SCFAs, indicating that viability of cells was not affected by experimental conditions. Furthermore, slight proliferation of PBMCs following LPS exposure in all samples indicated that the proliferation was mainly due to stimulation effect of LPS and that the SCFAs only promoted the LPS activated cell proliferation. The induction of cell proliferation indicate that LPS was able to trigger cell response and initiate the immune activity in PBMCs, involving B lymphocytes, T lymphocytes, dendritic cells, natural killer

cells, monocytes, and macrophages. This was expected as LPS is known to induce a macrophage-dependent immune response through activation of NF-κB transcription factor, subsequently enhancing proliferation and release of immune factors from PBMCs (Martich, Boujoukos, & Suffredini, 1993; Sharif, Bolshakov, Raines, Newham, & Perkins, 2007). In our study, acetate, propionate and butyrate did not cause apoptosis in PBMCs but promoted the proliferation of LPS-stimulated PBMCs. Different effects of SCFAs on proliferation of PBMCs and other similar cells have been highlighted (Meijer et al., 2010). A study on the effect of SCFAs in mouse macrophage cell line RAW264.7 reported that viability of cells was not affected when incubated with 0 - 1.2 mM SCFAs (Liu et al., 2012). Similarly, an earlier study demonstrated that unstimulated PBMCs were not affected by SCFAs at a physiological level (Cox et al., 2009). On the other hand some studies showed that butyrate caused apoptosis in antigen stimulated T cells and macrophages (Bailon et al., 2010; Kurita-Ochiai, Fukushima, & Ochiai, 1999). For example, 2 mM of butyric acid induced inhibition of proliferation after concanavalin-A stimulated porcine PBMCs (Weber & Kerr, 2006). Another study revealed that lymphocytes proliferation was inhibited due to 1.5 mM of butyrate but not acetate or propionate at the same concentration (Cavaglieri et al., 2003). These different effects of SCFAs might be due to the using of different concentrations, sources of PBMCs and method of stimulation (Meijer et al., 2010).

4.2. Effect of SCFAs on cytokine release

Lymphocytes besides inflammatory cells are involved in immune and inflammatory responses. They interact with each other via release of cytokines and expression of cytokine receptors in response to stimuli (Gruys, Toussaint, Niewold, & Koopmans, 2005). Cytokines can act as positive or negative regulators of immune responses and maintain lymphocyte balance. Nevertheless, the activity of cytokines are dependent upon each other along with environmental signals and receptor expression on effector cell surfaces (Scheller, Chalaris,

294 Schmidt-Arras, & Rose-John, 2011). Factors affecting cytokine network may subsequently
295 influence the inflammatory or immune response. Among these factors, SCFAs which have
296 been recognized as regulators of immune response through different mechanisms, such as
297 directly affecting the immune cells by binding to specific receptors GPCR (Le Poul, et al.,
298 2003), inhibition of HDAC (Davie, 2003), suppression of activation of the transcription
299 factor NF-κB (Segain et al., 2000) and revelling different anti-inflammatory activities via
300 regulation of cytokines secretion by immune cells (Cox et al., 2009; Saulnier, Spinler,
301 Gibson, & Versalovic, 2009).

302 The current study highlights the effect of SCFAs on secretion of cytokines that are involved
303 in T cell differentiation into either Th17 or Treg cells besides the main cytokines that are
304 released by Th17 and Treg. Stimulation of PBMCs with LPS alone lead to an increased
305 production of IL-6 and TGF-β as LPS is a known stimulant to induce the secretion of pro-
306 inflammatory cytokines (Jansky et al., 2003; Martich et al., 1993; Sharif et al., 2007). The
307 combination of LPS and SCFAs particularly butyrate decreased IL-6 and marginally reduced
308 TGF-β1. These effects were similar for either SCFAs added to 24 h pre-stimulated PBMCs or
309 at 0 time of stimulation (Table 1 and Fig. 3). The reduction of IL-6 and slightly reduced TGF-
310 β1concentration in the pre-stimulated PBMCs was probably due to the neutralizing and
311 inhibiting effects of SCFAs on pro-inflammatory condition in stimulated PBMCs. This might
312 have mediated the secretion of anti-inflammatory cytokines that likely act as immune
313 regulator of pro-inflammatory cytokines (Dinarello, 1997; Opal & DePalo, 2000; Sultani,
314 Stringer, Bowen, & Gibson, 2012). The effect of SCFAs on immune cells depends on the
315 activation status and differentiation stages of effector cells (Cox et al., 2009). Furthermore,
316 incubation of PBMCs in the presence of SCFAs without LPS did not induce changes in the
317 release of tested cytokines an indication that the physiological concentrations of SCFAs had
318 no obvious effects under our study conditions unless the cells were primed with LPS.

TGF-β1 has verity of functions, the exposure of PBMCs to TGF-β1 can generate a variety of cellular processes including inhibition of proliferation, differentiation, migration and apoptosis (Sanchez-Capelo, 2005). TGF-β1 regulates many other growth factors and plays a role in naive cell differentiation based on its concentration and other cytokine environment. TGF-β1 in the presence of IL- 6 , IL-1β, IL-21 or IL-23 drive cell differentiation to Th17 cells, subsequently releasing more pro-inflammatory factors (Yoshimura, Suzuki, Sakaguchi, Hanada, & Yasukawa, 2012; Liang Zhou, Chong, & Littman, 2009; Liang Zhou et al., 2007). Little is known about effects of SCFAs on release of TGF-β1 in human PBMCs, in our study important pleiotropic cytokine, TGF-β1 was increased significantly in the presence of LPS but only slight decrease with SCFAs. Increase in the concentration of IL-6 along with TGF-β1 could trigger differentiation of $CD4^+$ to Th17 cells. The induction of Th17 cells could be related to the down-regulation of IL-6 but not TGF-β1 since the level of TGF-β1 was not significantly affected by SCFAs. Consistent with our study SCFAs have been reported to supress pro-inflammatory mediators such as TNF-α, IL-6 and enhance the release of anti-inflammatory cytokine IL-10 (Meijer et al., 2010; Park, Lee, Lee, Kim, & Kim, 2007). Pleiotropic cytokines TGF-β1 plays important role in regulation of immune response by acting with other cytokines such as IL-2 and IL-10 to promote expression and activation of Treg cells, and released more IL-2 and IL-10, consequently up-regulating anti-inflammatory condition and ameliorates inflammation (Taylor, Verhagen, Blaser, Akdis, & Akdis, 2006).

4.3. Differentiation of $CD4^+$ cells

Based on the cytokine milieu, activated naïve T helper cells may be differentiated into Th1, Th2, Th17 or Treg phenotypes with different effector roles and cytokine profiles (Broere, Apasov, Sitkovsky, & van Eden, 2011). IL-12 milieu skews $CD4^+$ T helper cells to Th1, IL-4 skews $CD4^+$ T helper cells to Th2, TGF-β1 skews $CD4^+$ T helper cells to Treg and IL-6 with TGF-β skews $CD4^+$ T helper cells to Th17 (Afzali, Lombardi, Lechler, & Lord, 2007;

Kimura & Kishimoto, 2010; Ramgolam, Sha, Jin, Zhang, & Markovic-Plese, 2009). Furthermore other cytokines also play a role in cell differentiation and activation such as IL-2 which act with TGF-β1 to drive $CD4^+$ T cells to Treg cells (Campbell & Koch, 2011; Zheng, 2013; Ziegler & Buckner, 2009) and TGF-β with IL-1β, IL-21, and IL-23 are implicated in promoting human Th17 differentiation (T. Korn, Bettelli, Oukka, & Kuchroo, 2009; Ramgolam et al., 2009; Yang et al., 2008; Zheng, 2013; Ziegler & Buckner, 2009). In our study the, down regulation of Th17 cells may due to decline of IL-21 and IL-23 rather than reduction of TGF-β which was relatively stable in our study condition (Fig. 3).

Treg cells and Th17 cells are two subtypes of $CD4^+$ cells. They play opposing roles in autoimmune inflammatory diseases and immune tolerance, although they share a common differentiation pathway. Imbalance of Treg and Th17 has been established in several autoimmune diseases (Ji et al., 2012). Recent studies in human and mouse $CD4^+$ T cells show dichotomy in the generation and differentiation of Treg cells and Th17 cells (Kimura & Kishimoto, 2010; Zhu, Yamane, & Paul, 2010). The TGF-β1 signalling is described as the co-expression of Foxp3 and RORγ-t (L. Zhou et al., 2008). This signalling depends on other immune factors. For example, TGF-β1 alone enhances Foxp3 expression and inhibits RORγ-t activity, whereas combination of TGF-β1 with either IL-1β and IL-23 or IL-21 and IL-6 drives human Th17 differentiation (de Jong, Suddason, & Lord, 2010). This indicates the inner correlation between Th17 and Treg cells through the cytokine milieu (Ji et al., 2012).

SCFAs are supposed to have a regulatory influence on inflammatory disorder and ameliorate inflammation in some intestinal inflammatory disorders (Cox et al., 2009). This might be mediated through modulation of cytokine milieu in the medium and expression of Treg and Th17. Our finding exhibited that Foxp3 and RORγ-t expression in T lymphocytes subset population resulted after incubation of LPS stimulated PBMCs with different SCFAs. The stimulation of PBMCs with LPS elicited Th17 promoting cytokines mainly TGF-β1 and IL-6

leading to increased frequency of Th17 and the subsequent release of IL-17 and IL-21. However in the presence of SCFAs, Th17 cell differentiation was likely supressed and favoured cytokine environment for FoxP3 regulatory cell induction leading to enhanced FoxP3 expression. Butyrate was more effective in this regard than acetate and propionate. These findings are consistent with studies suggesting that butyrate showed strong anti-inflammatory properties (Cavaglieri et al., 2003; Liu et al., 2012; Meijer et al., 2010; Tedelind et al., 2007) Furthermore, Arpaia et al. (2013) and Furusawa et al. (2013) reported that butyrate promotes the regulation of intestinal Treg generation. However, the effect of SCFAs on T cell phenotypes need more studies.

5. **Conclusion**

SCFAs may have *in vitro* anti-inflammatory and immune regulatory effects through induction of Treg cells and production of anti-inflammatory cytokines. Butyrate showed more regulatory effect than propionate and acetate respectively. Our finding indicates that, SCFAs may have regulatory properties on inflammatory processes via the balance of Th17/Treg cells and pro and anti-inflammatory cytokines.

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List of figures:

Fig. 1.
MTS assay examining the effects of SCFAs on the viability and proliferation of PBMCs. Cells were sub-cultured in 96 well plates at initial density of 10^3 cells per 100 µL with or without SCFAs and/or LPS and incubated for 48 h. PBMCs in growth media was set as control. Cells viability (%) = (OD of SCFAs treated sample / OD of control sample) ×100. Ace = acetate, Pro = propionate, But = Butyrate, PBMCs = Peripheral blood monocular cells. Results are expressed as mean of five independent experiments with error bars showing the standard deviation, ($p < 0.05$).

Fig. 2.
Nitric oxide (NO) concentration (µM) in supernatants of stimulated PBMCs (1×10^6 cells/ mL) cultures. PBMCs were sub-cultured for 48 h either in the presence of LPS (5 µg/mL), LPS with each SCFA (acteate, propionate or butyrate) or LPS with the mixed SCFAs. RPMI media only was used as a control. Supernatants were collected after 24 h and 48 h and the level of NO was determined by the Griess reaction. NO levels were significantly different ($p < 0.05$) from the control values (bars with different letter). NO = Nitric oxide; LPS = Lipopolysaccharide; Ace = acetate; Pro = propionate; But = butyrate; SCFAs = short chain fatty acids. Data represent the mean of five independent experiments with error bars showing the standard deviation.

Fig. 3.
Cytokine concentrations of IL-1β (**A**), IL-2 (**B**), IL-6 (**C**), IL-10 (**D**), IL-17 (**E**), TGF-β1 (**F**), IL-21 (**G**) and IL-23 (**H**) in supernatant after PBMCs (1×10^6 cells/mL) were sub-cultured with either acetate, propionate, butyrate or mixed SCFAs for 24 h and 5 µg/mL. LPS was added to each well and incubated for a further 24 h or 48 h. PBMCs with LPS only was set as control. Data represent the mean of five independent experiments with error bars showing the standard deviation, (bars with different letter are significantly different ($p < 0.05$)). SCFAs = short chain fatty acids; IL = interleukin; TGF = transforming growth factor; Ctrl = control, LPS = Lipopolysaccharide, Ace = acetate, Pro = propionate, But = butyrate, Mix = Mixed SCFAs.

Fig. 4.
Expression of activation marker CD25 on T lymphocytes, plots were gated on CD3 (**A**), percentage of induced $CD25^+$ forkhead box protein 3 ($FoxP3^+$) **(B),** and induction of ROR-γt expressing T helper type 17 (Th17) (**C)** by PBMCs in response to LPS and/or SCFAs.

Table 1

Cytokine levels (pg/mL) produced by PBMCs co-cultured with LPS only for 24 h then SCFAs were added and the cells were incubated for another 24 h.

Cytokines	T(h)	Ctrl	LPS	Ace	Pro	But	Mix
IL-β1	24	55.04 ±6.3	1587.1 ±203.8	1571.7 ±130.2	1275.2 ±51.2	1601.1± 105.4	1620.1 ±33.0
	48	53.13 ±5.8	1828.2 ±166.4	1031.7 ±35.5	1121.2 ±48.4	992.5 ±166.3	1086.3 ±143.0
IL-2	24	17.33 ±0.5	285.13 ±5.1	291.32 ±12.2	287.23 ±8.5	290.1 ±41.7	289.21 ±6.1
	48	15.46 ±1.3	346.21 ±21.1	225 ±31.5	234 ±19.8	201.3 ±32.2	222 ±7.6
IL-6	24	59.36 ±3.3	1082.07 ±20.6	1001.21 ±25.7	1013 ±5.1	1014.12 ±83.3	997.39 ±64.6
	48	71.41 ±5.2	1097.13 ±11.9	831.3 ±32.4	942 ±25.1	612 ±32.9	741 ±46.2
IL-10	24	71.12 ±4.2	76.18 ±3.6	78.3 ±13.3	88.1 ±1.2	87.23 ±4.8	76.91 ±3.5
	48	77.34 ±3.2	74.75 ±2.8	181.2 ±6.4	173.15 ±8.1	26 ±12	263 ±20.8
IL-17	24	74.13 ±5.3	403.17 ±34.1	423.1 ±8.7	411.1 ±21.1	435.2 ±23.0	429.19 ±46.4
	48	78.41 ±1.9	432 ±14.1	107.4 ±2.8	172 ±5.3	121 ±10.5	133 ±16.8
IL-21	24	231.12 ±14.4	371.41 ±6.9	372.42 ±95.9	358 ±18.5	364.3 ±28.0	361.1 ±17.1
	48	236.71 ±12	391.35 ±19.9	62.21 ±3.3	117.7 ±5.9	61.5 ±9.4	75.13 ±4.0
IL-23	24	81.28 ±3.8	1271 ±89.8	1237 ±175.1	1211.5 ±81.1	1301 ±178.4	1291 ±38.3
	48	86.30 ±3.2	1295 ±116.0	920 ±34.1	989 ±32.1	931.2 ±11.7	1011 ±26.8
TGF-β1	24	161.1 ±9.1	1751 ±127.5	1723 ±108.2	1715 ±81.1	1695.4 ±17.7	1699 ±116.5
	48	156.3±4.1	1950±38.4	1710±46.1	1608±135.6	1651±31.5	1558±174.0

IL = interleukin; TGF-β1 = transforming growth factor beta 1; Ctrl = control (PBMCs without stimulation), LPS = Lipopolysaccharide, Ace = acetate, Pro = propionate, But = butyrate, Mix = Mixed SCFAs.

Fig. 1.

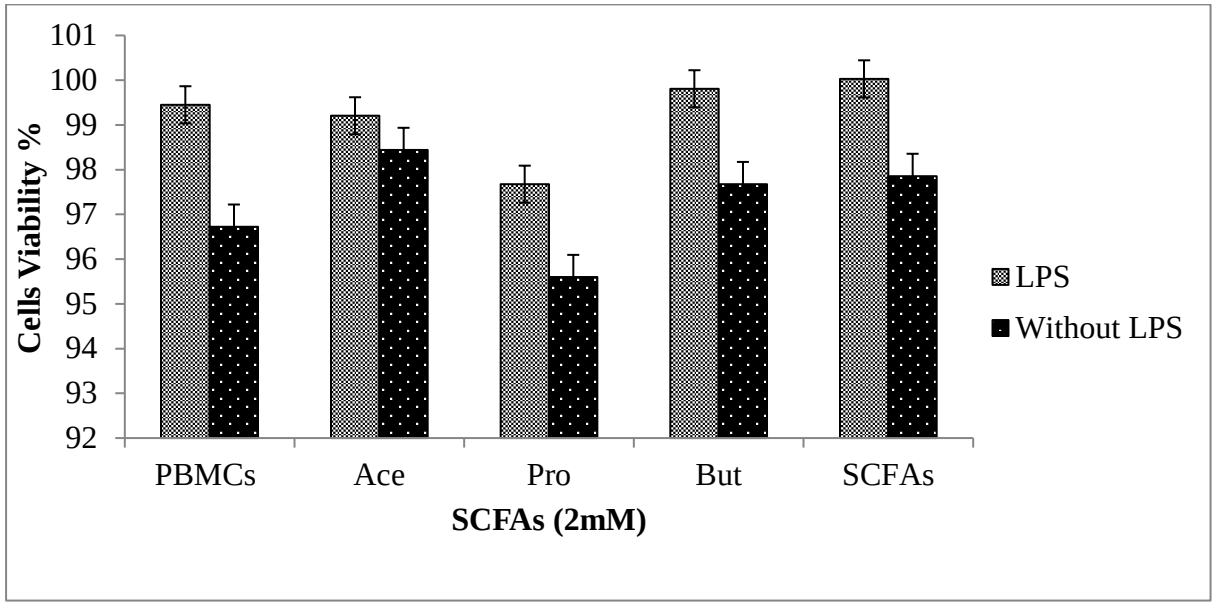
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Fig. 2.

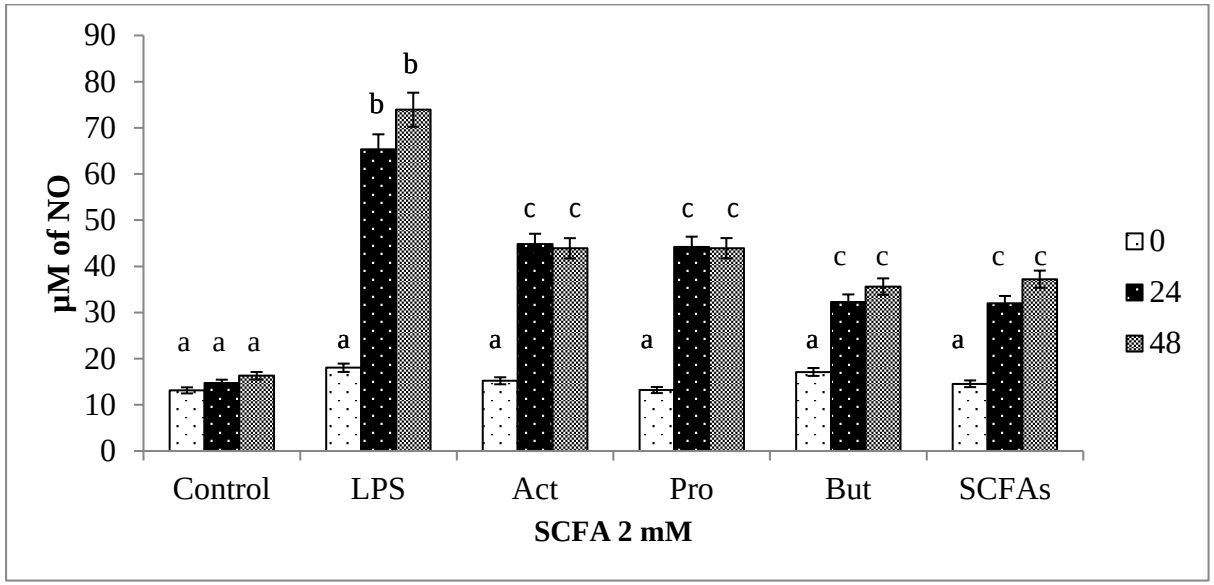
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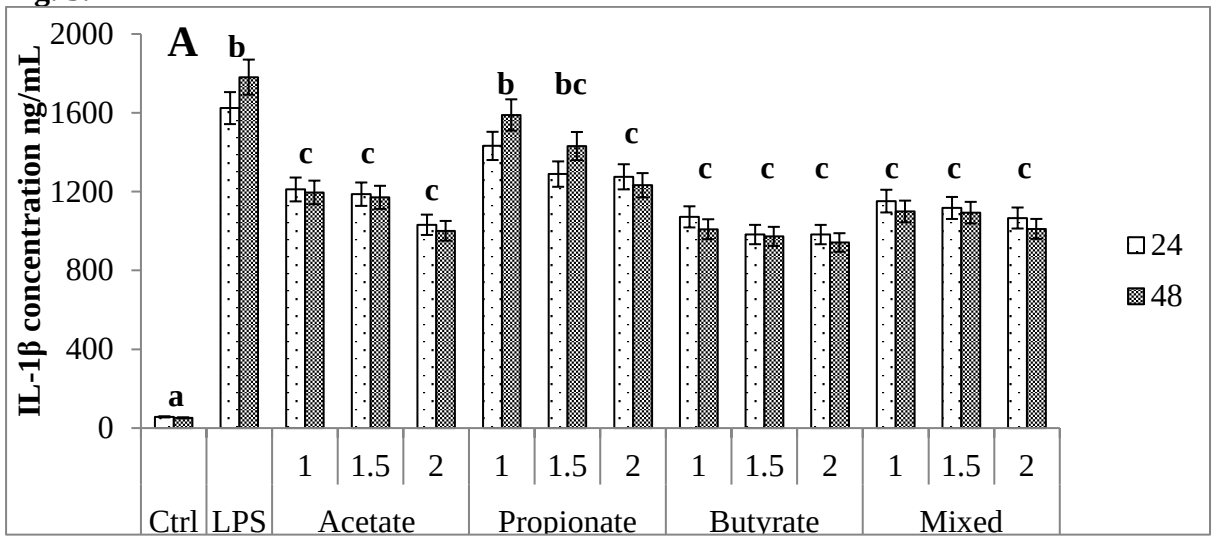
Fig. 3.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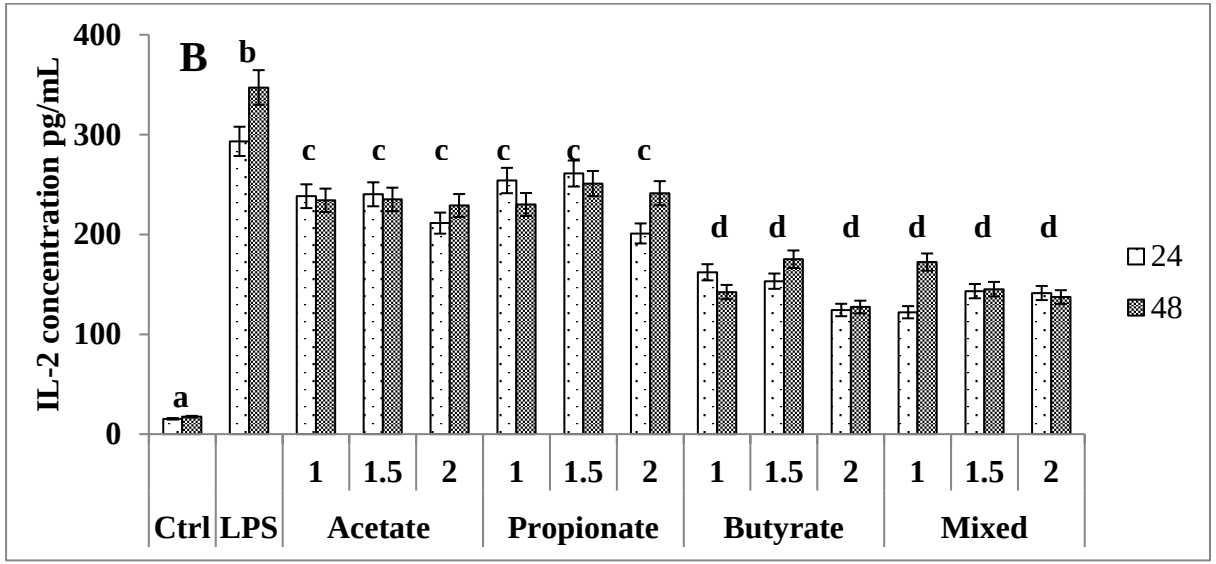
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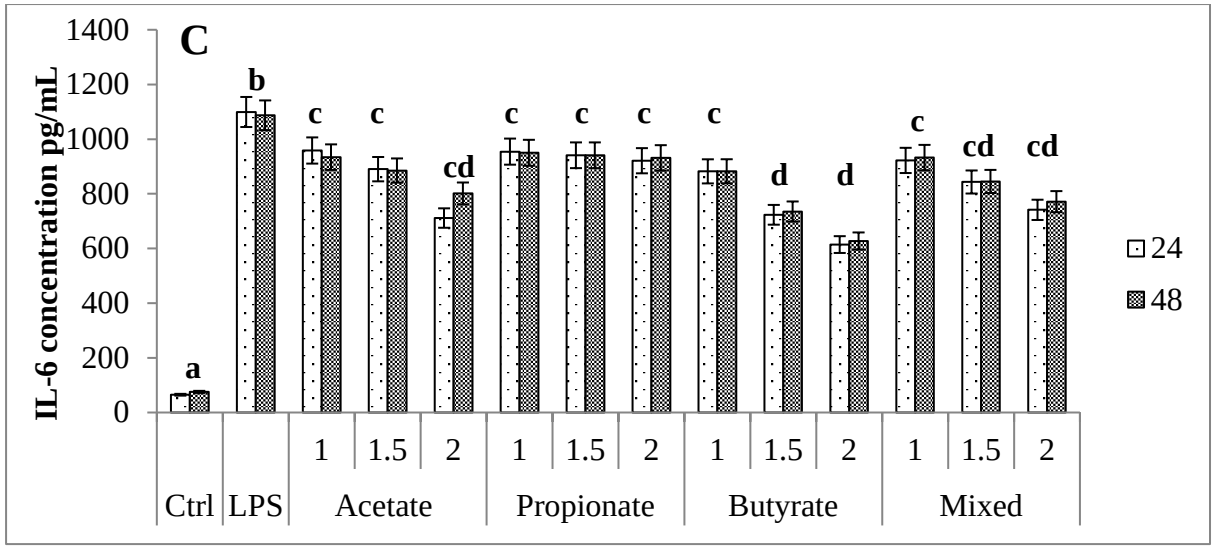
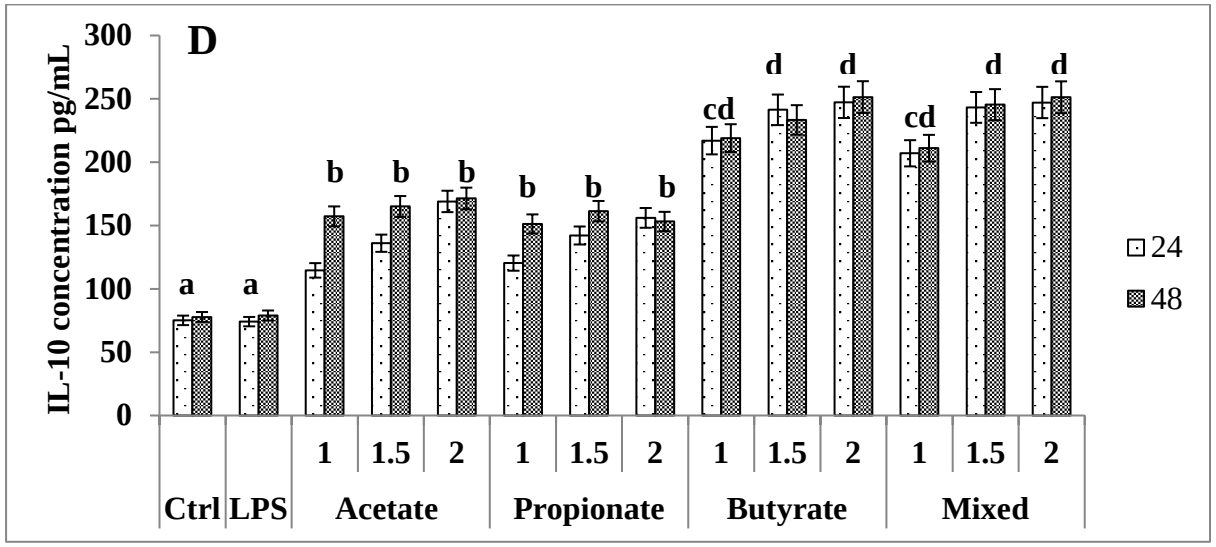
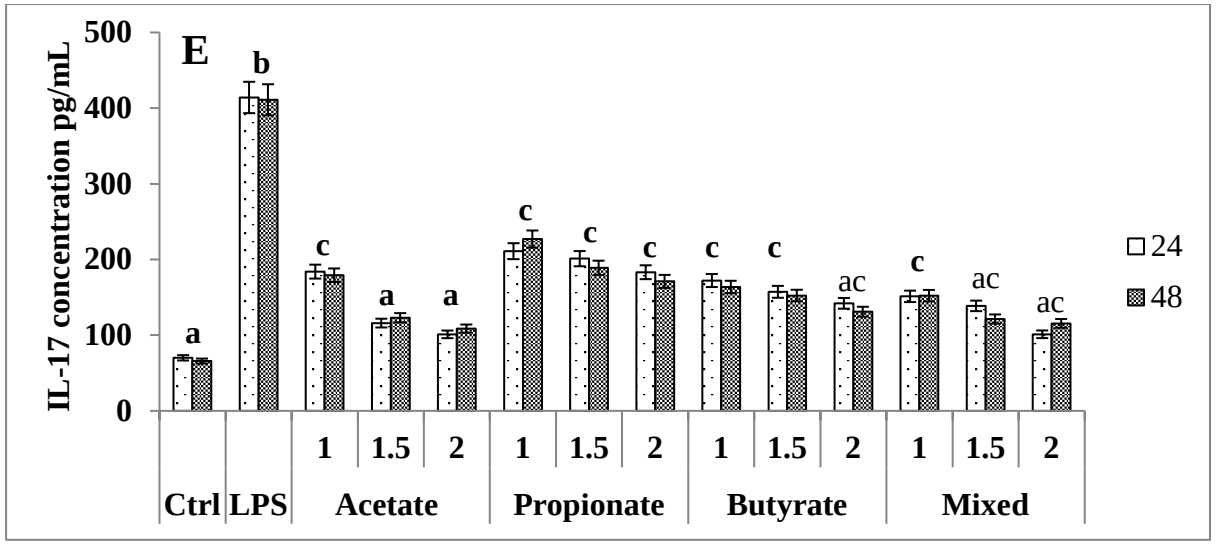
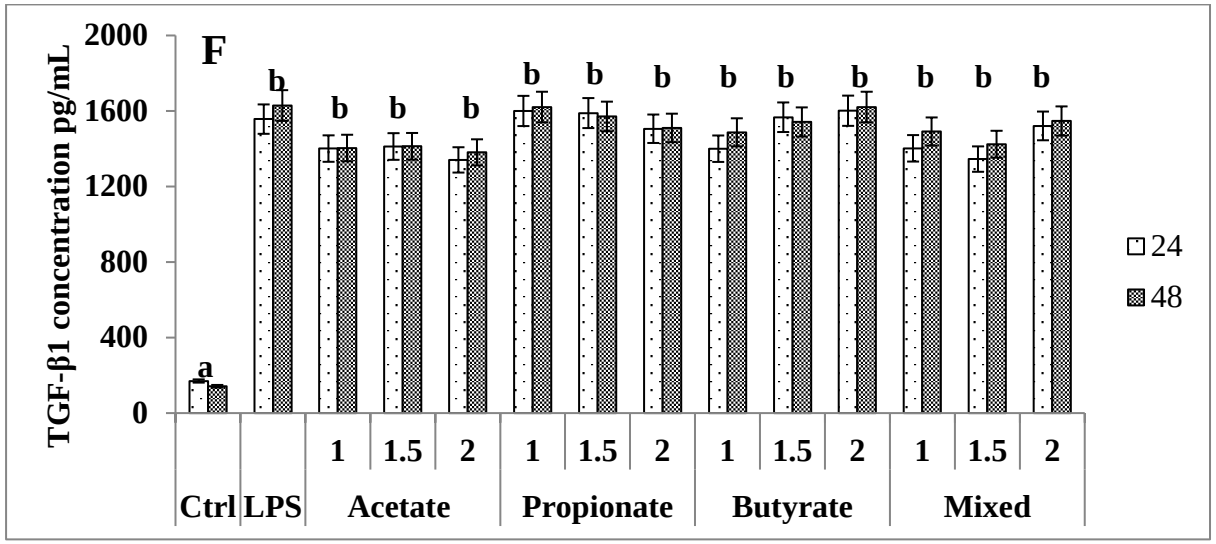
24
48


C
IL-6 concentration pg/mL
1400
1200
1000
800
600
400
200
0
a
b
c
c
cd
c
c
c
c
d
d
c
cd
cd
1
1.5
2
Ctrl
LPS
Acetate
Propionate
Butyrate
Mixed
24
48


D
IL-10 concentration pg/mL
300
250
200
150
100
50
0
a
a
b
b
b
b
b
b
cd
d
d
cd
d
d
1
1.5
2
Ctrl
LPS
Acetate
Propionate
Butyrate
Mixed
24
48


E
IL-17 concentration pg/mL
500
400
300
200
100
0
a
b
c
a
a
c
c
c
c
c
ac
c
ac
ac
24
48
1
1.5
2
Ctrl
LPS
Acetate
Propionate
Butyrate
Mixed

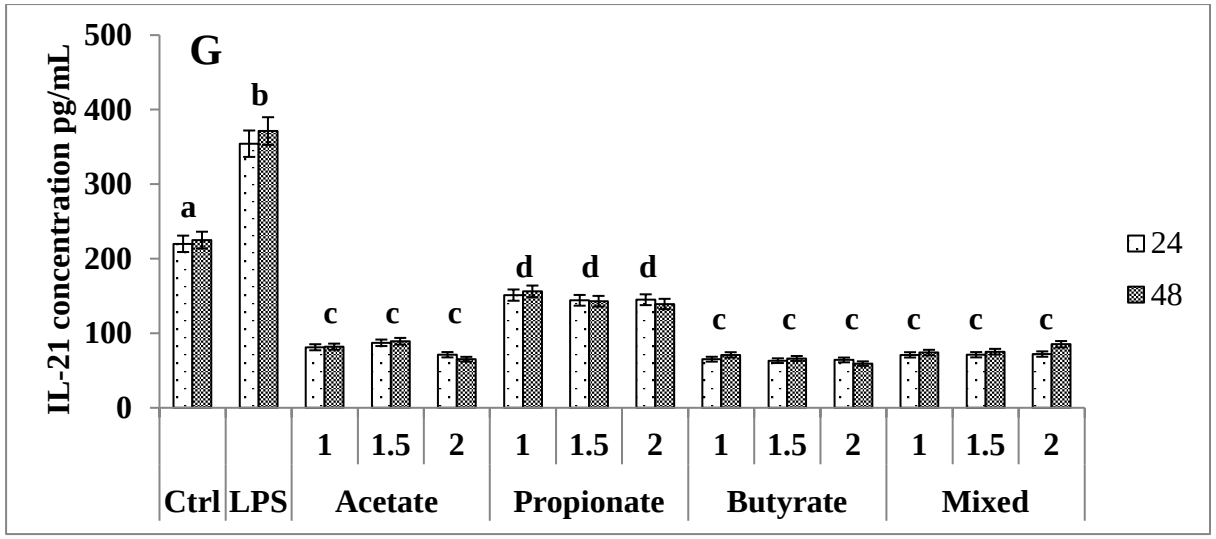
666


F
TGF-β1 concentration pg/mL
2000
1600
1200
800
400
0
a
b
b
b
b
b
b
b
b
b
b
b
b
b
b
24
48
1
1.5
2
Ctrl
LPS
Acetate
Propionate
Butyrate
Mixed

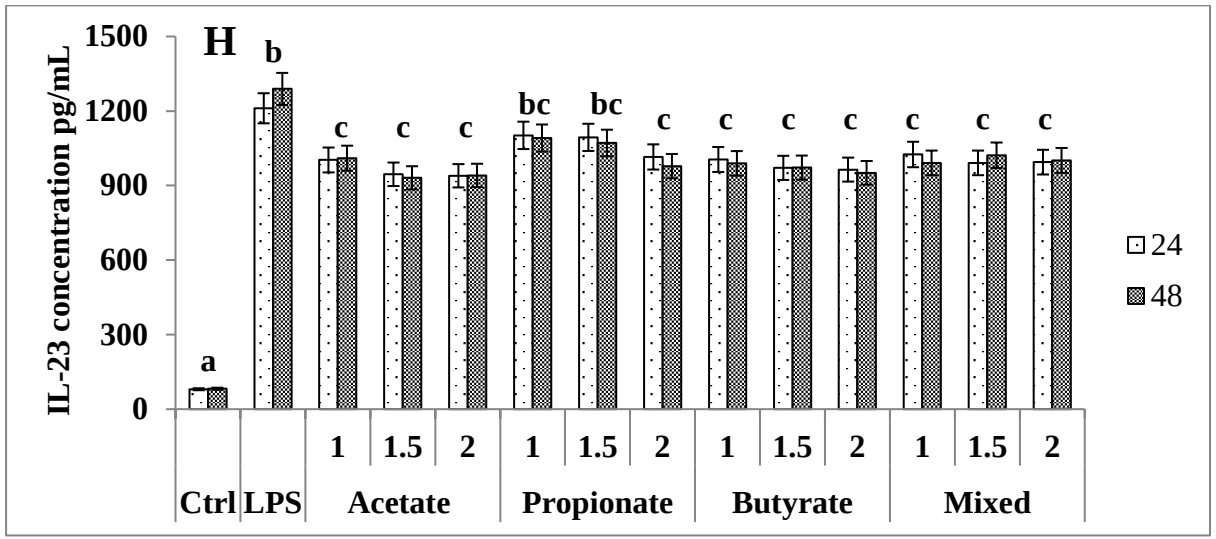
667

668

669

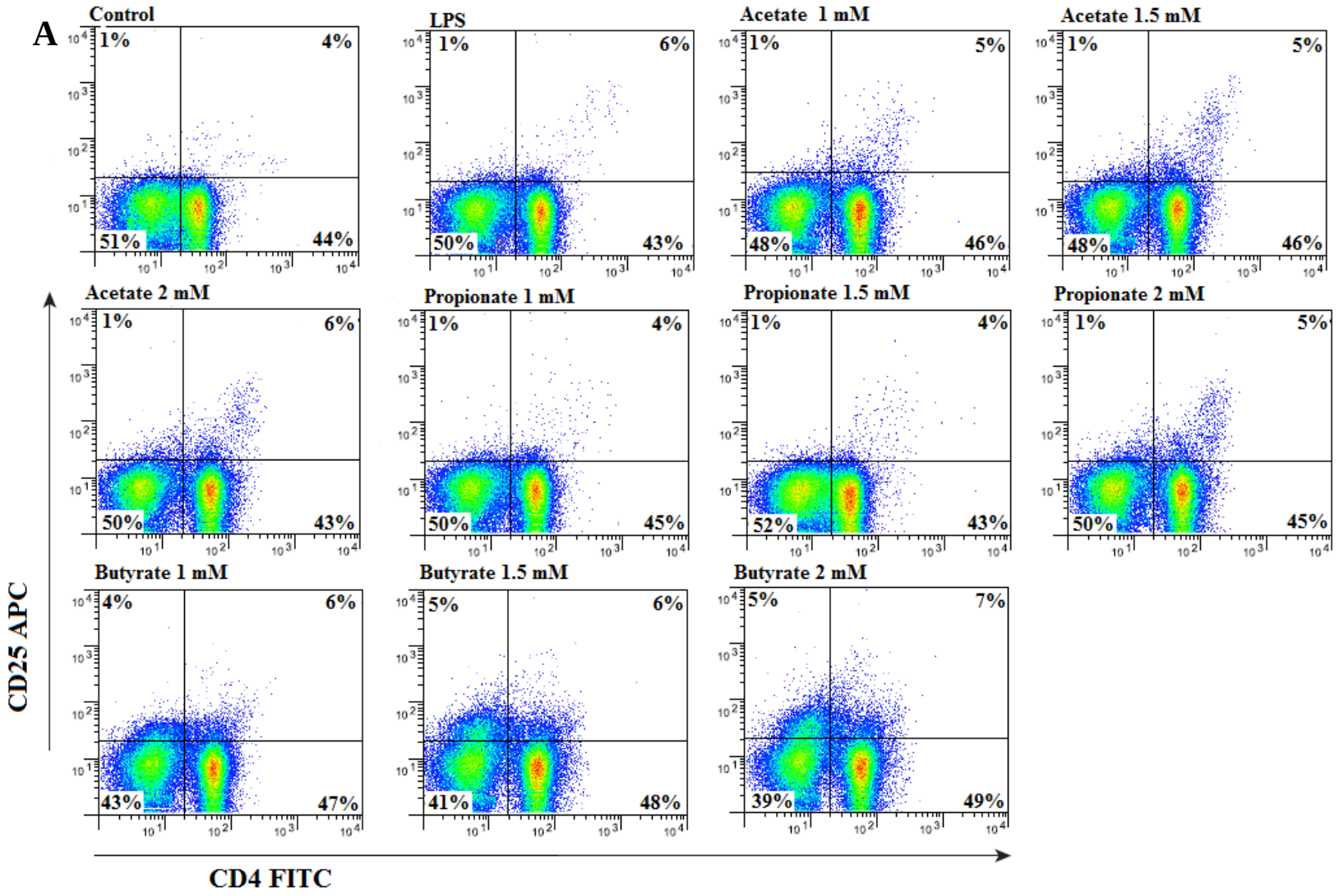

G
IL-21 concentration pg/mL
500
400
300
200
100
0
a
b
c
c
c
d
d
d
c
c
c
c
c
c
1
1.5
2
1
1.5
2
1
1.5
2
1
1.5
2
Ctrl
LPS
Acetate
Propionate
Butyrate
Mixed
24
48

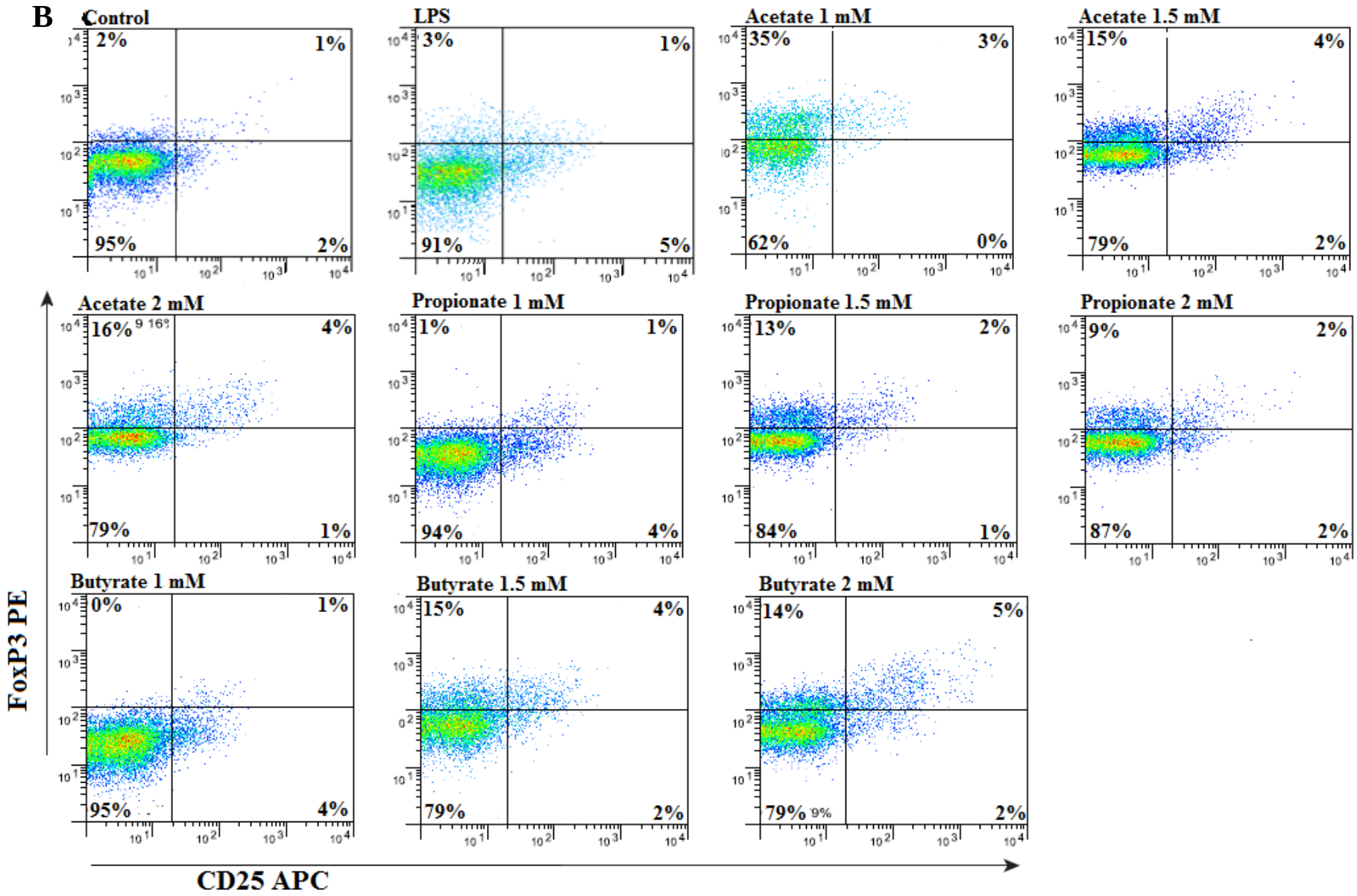
670

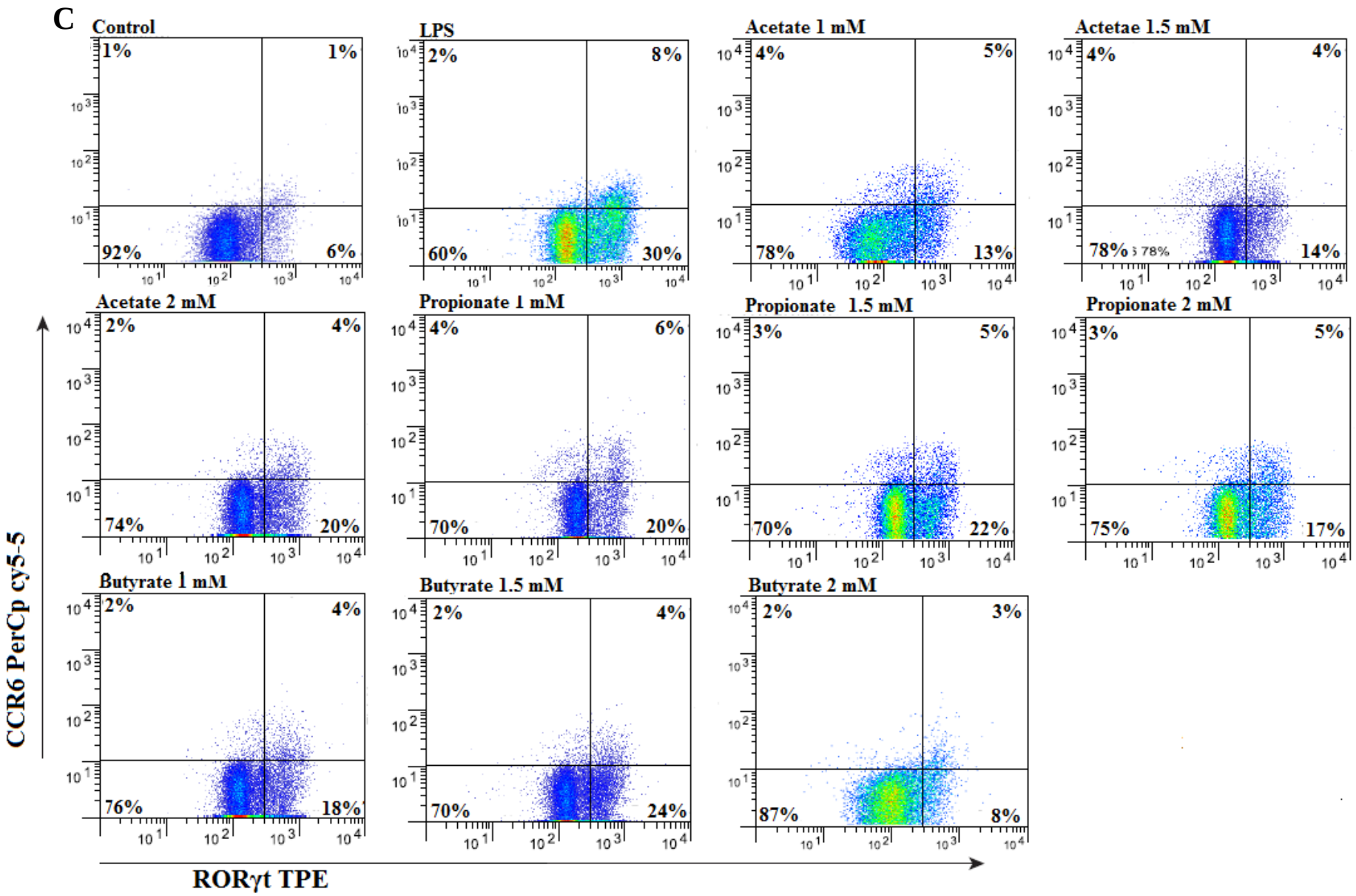

H
IL-23 concentration pg/mL
1500
1200
900
600
300
0
a
b
c
c
c
bc
bc
c
c
c
c
c
c
c
1
1.5
2
1
1.5
2
1
1.5
2
1
1.5
2
Ctrl
LPS
Acetate
Propionate
Butyrate
Mixed
24
48

671

Fig. 4.


A
Control
1%
4%
51%
44%
LPS
1%
6%
50%
43%
Acetate 1 mM
1%
5%
48%
46%
Acetate 1.5 mM
1%
5%
48%
46%
Acetate 2 mM
1%
6%
50%
43%
Propionate 1 mM
1%
4%
50%
45%
Propionate 1.5 mM
1%
4%
52%
43%
Propionate 2 mM
1%
5%
50%
45%
Butyrate 1 mM
4%
6%
43%
47%
Butyrate 1.5 mM
5%
6%
41%
48%
Butyrate 2 mM
5%
7%
39%
49%
CD25 APC
CD4 FITC


B
Control
2%
1%
95%
2%
LPS
3%
1%
91%
5%
Acetate 1 mM
35%
3%
62%
0%
Acetate 1.5 mM
15%
4%
79%
2%
Acetate 2 mM
16%
4%
79%
1%
Propionate 1 mM
1%
1%
94%
4%
Propionate 1.5 mM
13%
2%
84%
1%
Propionate 2 mM
9%
2%
87%
2%
Butyrate 1 mM
0%
1%
95%
4%
Butyrate 1.5 mM
15%
4%
79%
2%
Butyrate 2 mM
14%
5%
79%
2%
FoxP3 PE
CD25 APC


C
Control
1%
1%
92%
6%
LPS
2%
8%
60%
30%
Acetate 1 mM
4%
5%
78%
13%
Actetae 1.5 mM
4%
4%
78%
14%
Acetate 2 mM
2%
4%
74%
20%
Propionate 1 mM
4%
6%
70%
20%
Propionate 1.5 mM
3%
5%
70%
22%
Propionate 2 mM
3%
5%
75%
17%
Butyrate 1 mM
2%
4%
76%
18%
Butyrate 1.5 mM
2%
4%
70%
24%
Butyrate 2 mM
2%
3%
87%
8%
CCR6 PerCp cy5-5
RORγt TPE