

## Cytokine profile and induction of Th17 and Treg cells by human peripheral mononuclear cells after microbial exposure

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## Abstract

35 The immune-modulatory effects of probiotics were assessed following exposure of  
normal PBMC, cord blood cells, and the spleen-derived monocyte/macrophage cell line CRL-  
9850 to *Lactobacillus acidophilus* LAVRI-A1, *Lactobacillus rhamnosus* GG, EPS-producing  
*Streptococcus thermophilus* St1275, *Bifidobacterium longum* BL536, *Bifidobacterium lactis* B94  
and *Escherichia coli* TG1 strains. Cell production of a panel of pro- and anti-inflammatory  
40 cytokines following bacterial stimulation was measured, using live, heat-killed or mock GIT-  
exposed bacteria, and results show that i) all bacterial strains investigated induced significant  
secretion of pro- and anti-inflammatory cytokines from PBMC-derived monocytes/macrophages,  
(ii) cytokine levels increased relative to the expansion of bacterial cell numbers over time for  
cells exposed to live cultures. Bifidobacteria and *Streptococcus thermophilus* stimulated  
45 significant concentrations of TGF- $\beta$ , an interleukin necessary for the differentiation of Treg/Th17  
cells, and as such, the study further examined the induction of Th17 and Treg cells after PBMC  
exposure to selected bacteria for 96 hours. Data show a significant increase in the numbers of  
both cell types in the exposed populations, measured by cell surface marker expression and by  
cytokine production. Probiotics have been shown to induce cytokines from a range of immune  
50 cells following ingestion of these organisms. These studies suggest that probiotics' interaction  
with immune-competent cells produces a cytokine milieu, exerting immunomodulatory effects  
on local effector cells, as well as potentially inducing differentiation of Th17 and Treg cells.

**Keywords:** Probiotics, immunomodulation, Regulatory T-cells, Helper T-cell 17, Cytokines

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**Clinical and Experimental Immunology**

## 60 Introduction

Commensal bacteria in the intestinal lumen play an important role aiding digestion and synthesis of vitamins and nutrients. The composition of the gut bacterial population is relatively stable over time, but this profile can vary considerably between individuals [1]. This balance can be disturbed by dietary changes, stress and antibiotic treatment. However, a healthy balance can  
65 be re-established with probiotic supplementation, mainly consisting of *Bifidobacterium* species and selected lactic acid bacteria (LAB), which protect the host by excluding pathogenic bacteria and promoting immune modulatory responses from the gut epithelia [2].

T helper cell (Th) subsets are regulators of the adaptive immune response against infection. Th1-type cells produce cytokines which include, IL-2, TNF- $\alpha$  and IFN- $\gamma$ , activate  
70 macrophages and promote cell-mediated immunity, protective against intracellular infections. Th2-type cells produce a variety of anti-inflammatory cytokines including IL-1 receptor antagonist (IL-1ra), IL-4, IL-5, IL-6, IL-10, and IL-13 and promote humoral immune responses against extracellular pathogens [7]. Th17 cells are a subset of CD4<sup>+</sup> T cells that produce a pro-inflammatory cytokine IL-17. Th17 cells have recently been shown to play a critical role in  
75 clearing pathogens during host defense reactions and in inducing tissue inflammation in autoimmune disease [Korn et al., 2009; 11]. Regulatory T cells (Treg) are thought to be the master regulators of the immune response in both humans and rodents. Defects in the transcription factor forkhead box P3 (FoxP3), which defines the Treg lineage, results in multiple autoimmune diseases and atopy [15, 16], demonstrating the central role of FoxP3<sup>+</sup> CD4 cells in  
80 immune homeostasis..

The probiotic, *Lactobacillus (Lb) rhamnosus* GG, has been shown to influence Th2-, Th1-, and Th17-mediated disorders [20, 21, 22]. In addition, increases in FoxP3 mRNA expression in peri-bronchial lymph nodes have been noted upon administration of  
85 *Bifidobacterium lactis* Bb12 and *Lb. rhamnosus* GG, suggesting the induction of regulatory cells by these strains [23]. The important discovery that TGF- $\beta$  and IL-6 could promote Th17 differentiation from naive T cells [24] prompted studies that confirmed that Treg can also be generated *in vitro* by stimulation with TGF- $\beta$  in the absence of IL-6 [25, 26]. The remarkable balancing act of adaptive immunity to facilitate the targeted destruction of pathogens without excessive collateral damage to self is nowhere better exemplified than in the shared use of TGF-  
90  $\beta$  in controlling the newly described Th17 effector lineage and adaptive Treg development.

Probiotic bacteria can be potent inducers of cytokines, for example gram positive bacteria have been found to stimulate IL-12, while gram-negative bacteria tend to stimulate IL-10 production [32]. Several studies have demonstrated that selected probiotics are able to induce the production of pro-inflammatory cytokines by macrophages and Th1 cytokines by peripheral blood monocytes [33,34]. However, little is known about the effects of exposure time and bacterial state on the stimulation of cytokine production. As such, the aim of this study was to profile pro- and anti-inflammatory cytokines secretion from human PBMCs and the CRL-9850 cell line and the differentiation of Th17 or induced Treg cells following exposure to various strains of live, heat killed or gastrointestinal tract (GIT) simulated bacteria.

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## Materials and methods

### *Bacteria and cell lines*

*Lb. acidophilus* LAVRI-A1, *Bifidobacterium (B.) lactis* B94, and *Lb. rhamnosus* GG (LGG), were kindly provided by DSM Food Specialties (Moorebank, NSW, Australia), and Vaalia Parmalat Australia Ltd (South Brisbane, Queensland, Australia) respectively. Exopolysaccharides-producing *Streptococcus (S.) thermophilus* St1275, *B. longum* BL536, and pathogenic, *Escherichia (E.) coli* TG1 used as a Gram-negative control strain, were supplied by the culture collection of Victoria University (Melbourne, Australia). Strains were stored at -80°C in 40% glycerol. Sterile 10 mL aliquots of de Man Rogosa and Sharpe (MRS) broth (Sigma Chemical Co., St Louis, USA) supplemented with 0.05% L-cystein.HCl were inoculated with 10<sup>7</sup> colony-forming units (CFU)/mL of LAVRI-A1 and LGG and were incubated at 37°C for 18 h. For the propagation of *E. coli* and St1275, 10<sup>7</sup> CFU/mL of either strain was used to inoculate 10 mL tryptic soy broth (BHI, Difco Laboratories, Sparks, MD, USA) or M17 broth (Amyl Media, Dandenong, Australia) respectively [35]. Following two successive transfers to fresh 10 mL broth preparations, bacteria were grown for 18 hours log phase growth. Cultures were harvested at 1,360 x g for 30 min at 4°C. To heat kill, samples were incubated at 80°C for 30 minutes. GIT simulated samples were treated as described below. Following these manipulations, preparations were centrifuged and the pellet resuspended in PBS. Strains were washed three times in PBS and subsequently frozen at -80°C in aliquots of 10<sup>7</sup> CFU/vial in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 1% L-glutamine (Sigma).

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The spleen-derived human CRL-9850 cell line was purchased from the American Type Culture Collection (ATCC, Manassas VA, USA). Cells were grown in ATCC complete growth medium supplemented with 1% antimycotic solution (Sigma).

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#### *Simulated gastrointestinal digestion of bacteria*

The survival of gram-positive LAB and gram-negative bacteria in the gastrointestinal tract was investigated by simulating the physiological secretion of gastric acid and bile, in the stomach and the small intestine respectively. The method described in previous studies [4, 36] was used with some modifications as described. To simulate bacterial digestion in the stomach, 130 distilled-deionised water (40 mL) was added to 0.3 g of bacterial pellet, and the pH was adjusted to 2.0. Then, 0.25 g of freshly prepared pepsin solution (4% pepsin A (E.C. 3.4.23.1; Sigma, St. Louis, MO, USA) in 0.1M HCl, pH 2.0 was added and the volume was brought to 100 mL. Following incubation at 37°C for 2 h in a shaking water bath, the sample was incubated on ice, 135 10 min, to stop pepsin digestion. For the subsequent intestinal digestion the pH of the gastric digests was brought to pH 5.2, then 0.6 g of freshly prepared pancreatin-bile extract mixture (pancreatin (0.04 g), from porcine pancreas, plus bile extract (0.25 g) (Sigma) dissolved in 10 mL of 0.1M NaHCO<sub>3</sub>, pH 5.2 was added and incubated for an additional 2 h in the 37°C shaking water bath. After a subsequent 10 minute incubation on ice, the pH was adjusted to 7.2 and 140 samples were centrifuged (1,360 x g for 15 min, 4°C), pellets washed in PBS, before resuspending in 30 mL PBS.

#### *Enumeration of bacterial cells*

For enumeration of bacterial cell number, 1 mL of each freshly prepared culture (live 145 (untreated), GIT and killed) was 10-fold serially diluted and plated onto tryptic soy agar (*E. coli*), M17 agar (St1275), MRS agar (LAVRI-A1 and LGG) and MRS agar supplemented with 0.05% L-cystein.HCl (bifidobacteria), and anaerobically incubated for 72 h at 37°C [37]. For all bacterial strains, standard growth curves were produced by plotting optical density at 610 nm in MRS broth versus agar plate counts of freshly-prepared, serially-diluted cultures. These curves 150 were fitted with logarithmic expressions (in order to calculate viable bacterial counts in freshly-prepared cultures) of which each yielded  $r^2$  values of >0.985 (data not shown).

*Isolation of human peripheral mononuclear cells from buffy coat and cord blood using Ficoll gradient*

155 Human peripheral mononuclear cells were isolated from buffy coats (Australian Red Cross Blood Services (ARCBS), Melbourne, Australia) and cord blood (CB, Cord blood bank, Royal Children Hospital, Melbourne, Australia) by Ficoll paque gradient. PBMCs were isolated according to the methods described by de Roock et al., and Hesse et al. with minor modifications [38,32]. Briefly, buffy coats were diluted with an equal volume of PBS and  
160 layered on Ficoll-Paque Plus (GE Healthcare, Bio-Sciences, Uppsala, Sweden). Cells at the interphase were collected following centrifugation (680 x g, 25 min, 18°C) (Sorvall® RT7 centrifuge, DuPont, Newtown, Ct. USA). Blood lymphocytes were washed once in cold PBS, and following centrifugation (680 x g, 18°C, 10 min) the pellet was resuspended in 2 mL red blood cell lysis buffer (0.15 M NH<sub>4</sub>Cl, 0.01 M KHCO<sub>3</sub> and 10 μM EDTA Na<sub>2</sub>.2H<sub>2</sub>O) and  
165 incubated 2 min, room temperature. The volume was then adjusted to 30 mL using sterile PBS and centrifuged. Following two subsequent washes, the cell pellet was resuspended in IMDM (Sigma) supplemented with 10% fetal bovine serum (FBS, GIBCO, Mulgrave, Australia), and antimycotic solution (Sigma, 10mg/L).

170 *Stimulation of human PBMCs and CRL-9850 cells with bacteria and cytokine quantification*

PBMCs/CRL-9850 cells were plated in 6-well tissue culture plates (Corning, Sigma) at 5 x 10<sup>6</sup> cells/well and incubated at 5% CO<sub>2</sub>, 37°C for 24 h prior to stimulation with bacteria as described by Amrouche et al. [31]. Briefly, 10<sup>6</sup> freshly-prepared viable (live or GIT) or equivalent (~10<sup>6</sup> CFU/mL) heat-killed bacteria were added per 10<sup>6</sup> cells and co-cultured for 72 h  
175 at 5% CO<sub>2</sub>, 37°C. At 6, 12, 24, 48 and 72 h, 500 μL samples of the culture medium were collected and analysed for cytokine secretion by ELISA (BD) in accordance with the manufacturer's instructions. 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.

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*Cell staining and Flow Cytometry analysis*

Treg/Th17 populations were characterized following PBMC/bacteria co-culture. Briefly, 10<sup>6</sup> PBMC were co-cultured with either live or killed bacteria, lipopolysaccharides (LPS, Sigma)

185 or media alone, in a 24-well plate at 37°C in 5% CO<sub>2</sub> for 96 h, then cells were washed twice  
using FACS buffer (PBS + 2% FCS) and centrifuged at 500 x g for 10 min. PBMC were  
resuspended at 10<sup>6</sup> cells/mL, and surface marker staining was performed using fluorescein  
isothiocyanate (FITC) -labeled anti-human CD4, allophycocyanin -labeled anti-human  
190 CD25/CD3 (BD Pharmingen, California, USA), peridinin chlorophyll protein (PerCP) -labeled  
anti-human CD3 (Biolegend, San Diego, CA) and PerCP cyanine (Cy)5.5 -labeled anti-human  
CCR6 (CD196). Intracellular staining was done using phycoerythrin (PE) -labeled anti-human  
FoxP3/RORγt (BD Pharmingen and R&D systems, Minneapolis, USA respectively) according to  
the manufacturer instructions. Samples were read using a BD FACSCalibur, data acquired using  
CellQuest program (BD Biosciences), and analysis done using Gatedlogic 3.07 software (Inivai,  
Australia). Absolute numbers of Treg cells and Th17 cells were calculated as a percentage of the  
195 total lymphocyte number.

### Statistical analysis

All co-cultures were carried out in triplicate. Results obtained were analysed as a split  
plot in time design with 3 main factors: strains (6 levels) and treatments (3 levels) as the main  
200 plot and time (5 levels) as a subplot. The statistical evaluations of the data were performed using  
the General Linear Model [39]. Significant differences between treatments were tested by  
analysis of variance (ANOVA) followed by a comparison between treatments performed by  
Fisher's least significant difference (LSD) method, with a level of significance of p<0.05.

### 205 Results

*Cytokine secretion by PBMCs, cord blood and spleen-derived macrophage cell line following  
co-culturing with live bacteria*

Pooled PBMCs or CRL-9850 cells incubated with selected live bacteria for 48 and 72 h  
yielded cytokine levels as shown in Fig.1 a-c, Fig.2 a,b. Also shown are three individual donor  
210 cytokine profiles (48 or 72 h) as a representative of the 30 donor PBMCs investigated depicting  
varying cytokine levels detected between donors (Tables 1 a-c). A comparison of the 30  
individual donor PBMCs with the pooled donor PBMCs, shows significant differences of  
cytokine levels in line with previous results [40]. Even though some cytokines were not  
detectable from individual donors, substantial and significant production of all investigated

215 cytokines were recorded from pooled PBMC in response to LAB. All strains of bacteria had the  
capacity to induce pro- and anti-inflammatory cytokine production from the cell line and  
PBMCs, however the magnitude of production of each cytokine varied depending on the strain,  
as similarly reported by Wu et al. [27]. Generally, buffy coat sourced PBMC produced  
220 significantly higher ( $p < 0.05$ ) concentrations (100 – 8800 pg/mL) of cytokines compared to cord  
blood-derived PBMCs or CRL-9850 cells. In addition, cytokine production in the buffy coat  
PBMC was detectable from early culture (6h, data not shown) and maintained up to 72 hours,  
while cord blood PBMC and CRL 9580 cells showed a later appearance of cytokines in culture  
(48-72 hours, Fig. 2a,b), the delayed response likely due to a lack of established adaptive  
immune responses in cord blood [41]. Whilst pro-inflammatory cytokines were significantly  
225 produced in the supernatants for all treatments, anti-inflammatory cytokines such as TGF- $\beta$ , IL-  
6, and IL-10 were also detected. In the majority of cord blood samples, T-cell responses show an  
IL-10 or Th2-like pattern of cytokine production (Fig. 2a) [41, 42]. Previous studies have  
suggested that IL-10 may play a major role in influencing the activity of the placental  
trophoblast, which has been proposed as a key cell type in regulating foetal immunoprotection  
230 [43, 44].

#### *Cytokine secretion induced by mock GIT subjected bacteria*

The survival of bacteria subjected to conditions mimicking those in the GIT (e.g. low pH,  
exposure to enzymes and bile) was measured and compared to untreated bacteria growth. No  
235 significant differences were observed between the two sets of results, indicating the bacteria are  
able to withstand the harsh physiological conditions (Table 2) [4, 45]. Pro-inflammatory cytokine  
production was measured following co-cultured of GIT simulated bacteria with the different cells  
as above. In general, results showed cytokine production similar to that observed from live  
bacteria (Figs. 1a,b). Of all the bacterial strains assessed, St1275 induced the highest production  
240 of IL-12 from buffy coat PBMC (Fig. 1b). Conversely, when cultured with cord blood derived-  
PBMC, St1275 induced significantly ( $p < 0.05$ ) lower levels of IL-12 compared to other bacteria  
(data not shown). Again St1275 appeared to have stimulated significantly higher concentration  
of IL-17 in all GIT co-cultured from buffy coat derived-PBMCs but lower concentrations or no  
production with CRL9850 or cord blood derived-PBMCs (Figs. 1b,2b). *E. coli* induced IL-10  
245 secretion poorly from buffy coat PBMC. In contrast LAVRI-A1, B94, BL536, ST1275 and LGG



were found to stimulate high levels of IL-10 (Fig. 1b). From CRL9850 and cord blood derived-PBMCs only LAVRI-A1, LGG, B1536 and B94 induced significant ( $p < 0.05$ ) levels of IL-10 production (Fig. 2a).

#### 250 *Cytokine secretion induced by heat killed bacterial cells*

Killed bacteria were able to induce substantial levels of all cytokines from buffy coat PBMC (Fig. 1c). Strikingly, only IL-10 was seen to be induced in significant amount ( $p < 0.05$ ) when killed bacteria were incubated with the other cell types.

#### 255 *Induction of FoxP3 and ROR- $\gamma$ t expression in PBMCs by live/killed selected bacteria strains*

PBMC incubation with LAB resulted in enhanced expression of CD25 on CD4<sup>+</sup> T lymphocytes (Fig. 3) in line with Niers et al. [40]. To investigate whether treatment with lactobacilli or bifidobacteria lead to enhanced Th17 or Treg cell differentiation we assessed Th17/Treg populations in PBMC following 72 to 96 h of treatment with live or heat killed  
260 bacteria. In all cases, following 72 to 96 hours co-culture the number of Treg (CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup>) cells as a percentage of total PBMC increased substantially compared to untreated control cells, albeit to different levels (Figs. 4A (a,b)). BL536 and B94 were found to be the most potent live strains and LAVRI-A1, B94 and St1275 the most potent heat-killed strains at inducing FoxP3 expression. The capacity of live or killed bacteria to induce IL-17  
265 producing cells from PBMC was also investigated. As shown in Fig. 4B, the number of IL-17 expressing CD3<sup>+</sup> CD4<sup>+</sup> cells was substantially increased compared to control. Since Th17 cells typically produce IL-17 in culture, it was therefore likely that these cells were of the Th17 lineage. To confirm Th17 cell identity, extracellular marker CCR6 (CD196) and intracellular marker ROR- $\gamma$ t were subsequently used. The proportion of Th17 cells (CD3<sup>+</sup>CD4<sup>+</sup>CCR6<sup>+</sup>ROR- $\gamma$ t<sup>+</sup>)  
270 induced by live and for killed bacteria was increased 2.5-fold above control (Fig 4B (a,b)). with St1275 being the most potent strain ( $p < 0.01$ ). Interestingly, the induction of Th17 cells by the stimulation of PBMCs with *E. coli* or LPS were similar.

### **Discussion**

275 Probiotic bacteria are commonly marketed to aid digestion and optimise microbial balance in the GIT. The current studies assessed the capacity of probiotic bacteria to affect the

local cytokine production and regulatory cell populations among different cell types. In addition, the models used in these studies simulated the conditions that ingested micro-organisms face during transit through the GIT such as low pH, bile concentration, and enzymatic digestion to assess their effect on cell survival and the capacity to influence host immunoregulation [49, 4]. Our results demonstrated the bacteria were resistant to the extreme conditions faced in the gut in line with previous reports [4].

The current studies assessed the ability of common probiotics to induce cytokine production from PBMCs, cord blood cells and spleen-derived macrophages. The substantial concentrations of IL-2, IL-12, IL-17 and IFN- $\gamma$  produced by PBMCs in this study indicate the cells' potential to prevent/fight infection. LGG has been reported to aid in the prevention of atopic dermatitis in infants and as well as alleviate food allergy [53, 54]; if these effects are largely IL-12 driven, St1275, B94 and *E. coli* in our study may likely be as effective in their immunomodulatory effects. Miettinen et al. reported that LGG induced the production of pro-inflammatory cytokines such as IL-6, IL-12 and IFN- $\gamma$  but limited IL-10 from human PBMC [34]. On the other hand in our study, LAVRI-A1, LGG and bifidobacteria induced significantly higher concentrations of IL-10 from PBMCs compared to the pro-inflammatory cytokines, which makes these probiotic strains good candidates for management of autoimmune disorders.

In the current study we report that selected probiotics induced significant amounts pro-inflammatory cytokines including IL-2 which is a critical cytokine for clonal expansion of recently antigen-activated T cells and in regulatory T cell homeostasis [55]. Macrophage-produced IL-12 stimulates IFN- $\gamma$  production in T cells and natural killer cells, which accelerates the development of naive CD4<sup>+</sup> T cells into Th1-type cells [51]. Therefore, IL-12 is a key immunoregulator favouring Th1-type responses. However IFN- $\gamma$  in turn induces IL-12 production, which can cause a positive feedback loop of IFN- $\gamma$  and IL-12 production and can be detrimental, leading to uncontrolled cytokine production and possible shock [56]. IL-17 has recently been found to be elevated in the intestinal tissue and serum of patients with IBD and other autoimmune disorders [57]. In contrast, anti-inflammatory cytokines IL-4, IL-10 and TGF- $\beta$  were also found to be produced in significant concentrations by our healthy PBMCs with the co-culture of selected bacteria. These cytokines function to inhibit IL-12 and the production of other pro-inflammatory cytokines from antigen-presenting cells including macrophages as well by inducing expression of other co-stimulatory surface molecules and soluble cytokines [61].

Our findings show that all the selected bacteria, especially LAVRI-A1, LGG and bifidobacteria induced significant secretion of IL-10 and TGF- $\beta$ , which was in line with earlier reports on *L. acidophilus* and bifidobacteria [33, 62, 63]. In addition to its activity as a Th2 lymphocyte cytokine, IL-10 is also a potent deactivator of monocyte/macrophage pro-inflammatory cytokine synthesis [65]. TGF- $\beta$ 1 down regulates monocyte and macrophage activity in a manner similar to IL-10, albeit less potently [66]. It suppresses the proliferation and differentiation of T cells and B cells and limits IL-2, IFN- $\gamma$ , and TNF- $\alpha$  production. The severe and uncontrolled inflammatory reactions observed in the TGF- $\beta$ 1 knockout mouse attests to the physiologic role of TGF- $\beta$  as an endogenous anti-inflammatory cytokine [29].

Even though in this study gram-negative *E. coli* stimulated substantial amount of pro-inflammatory cytokines, the induction of pro- and anti-inflammatory cytokines with live gram-positive bacteria (including GIT simulated bacteria) on average, was significantly higher. Hessle et al. [32] reported that gram-positive bacteria appeared to stimulate IL-12 production and gram-negative bacteria preferentially stimulate IL-10 production. However, concordant with observations reported in Berg et al. [67] and in our study, gram-negative *E. coli* induced the secretion of significant concentrations of pro-inflammatory cytokines by PBMCs and the CRL-9850 cell line. While the mechanisms by which some bacteria induce the production of IL-10 are unclear, LPS of gram-negative bacteria may stimulate this anti-inflammatory response [67]. Compounds other than LPS in lactobacilli probably contributed to the ability of these probiotic bacteria to stimulate an anti-inflammatory cytokine response. Probiotic LAVRI-A1, LGG, B94 and BL536 induced substantial amounts of pro-and anti-inflammatory cytokines in line with previous studies [81] with the balance skewed towards the anti-inflammatory response in our study. A demonstration of the utility of this response is the finding that LGG reduced inflammation in Crohn's disease [70]. The human gut microbiota has been recently estimated to consist of at least 400 different species [68], and it is likely that the potency of each of these species to influence immune homeostasis is different. Indeed, cytokine profiles in co-cultures of bacteria with PBMC show marked differences between strains [40]. In addition, the effects of lactobacilli supplementation on experimental autoimmune encephalomyelitis have been shown to be highly strain dependent [69]. It is therefore conceivable that the contradicting results found in the human trials can be partly explained by differences in the immunomodulatory capacity of the strains used.

340 The fact that the killed bacteria in our study were inefficient in inducing substantial  
amounts of pro- and anti-inflammatory cytokines compared to live bacteria suggests that extra-  
and intra-cellular bacterial components as well as metabolites likely contribute to cytokine  
production [71]. Conceivably, a combination of certain bacterial fragments, metabolites  
produced *in situ*, and particular structural motifs may need to interact with receptors on  
monocytes to induce optimal cytokine synthesis [72, 31]. Cross et al. [73] and Macpherson and  
345 Harris [74] reported that live *lactobacilli* were more potent inducers of cytokine production in  
mammalian leucocytes compared to killed bacteria, similar to our findings. The results of the  
present study indicate that differential immuno-modulatory effects may exist between  
*Lactobacilli*, bifidobacteria and *S. thermophilus*, suggesting that these bacteria may be stronger  
boosters of host immunity. However in the case of St1275, the presence of EPS might have also  
350 influenced its ability to stimulate sustained and substantial levels of cytokines in the co-cultures.  
Exopolysaccharides from LAB have been claimed to participate in various regulatory processes  
such as immunomodulatory, cholesterol-lowering and anti-ulcer activities [5].

This study also investigated the differentiation of Treg and Th17 cells from PBMCs  
stimulated with the bacteria. TGF- $\beta$  has been shown to be involved in both Treg and Th17  
355 development. Animal models have demonstrated that at high levels of TGF- $\beta$ , Foxp3 expression  
is upregulated and Treg differentiation is induced, whereas at low levels of TGF- $\beta$ , IL-6 and IL-  
21 synergize to promote the differentiation of Th17 cells [88]. The identification of the  
transcription factor ROR- $\gamma$ t by intracellular and CCR6 extracellular staining confirms the  
differentiation of Th17 cells in the current experiment. Th17 cells induce a range of pro-  
360 inflammatory mediators that bridge the innate and adaptive immune response enabling the  
clearance of invading pathogens [89]. The balance between Treg and Th17 cells may be essential  
for maintaining immune homeostasis. Hence, therapeutic approaches that aim to re-establish  
homeostasis by increasing the number of Treg, while also controlling effector T cell populations,  
may prove effective in the treatment of autoimmune diseases whereas the reverse may also hold  
365 true for inflammatory diseases such as allergy.

In the current studies, the bacterial strains that induced high FoxP3 expression also  
stimulated the highest levels of the suppressive cytokine, IL-10 [38]. The mechanism of FoxP3<sup>+</sup>  
Treg induction in the co-cultures still remains unclear. TGF- $\beta$  appears to be a key cytokine in  
this induction, although IL-2 also plays an apparent and important role [92]. This was also

370 apparent in our study since IL-2 and TGF- $\beta$  were among the various cytokines released.  
Furthermore, we have shown that production of cytokines and induction of ROR- $\gamma$ t/FoxP3 cells  
were strain dependent, and differed depending on bacterial treatment (i.e. live or killed). Similar  
findings were reported previously [38] when strains of lactobacilli differed significantly in their  
capacity to induce FoxP3<sup>+</sup> regulatory cells *in vitro*, independent of the IL-10 production. The  
375 overall extent of induction of FoxP3<sup>+</sup> (Treg) and ROR- $\gamma$ t<sup>+</sup> (Th17) cells by the selected bacteria in  
our study showed a balance between these cells, representative of that found in a healthy donor  
[48]. Previously *Lb. acidophilus* strain LAVRI-A1 had no clinical effect on eczema [94];  
however this strain may be effective for other inflammatory disorders, since the current study  
shows a moderate induction of FoxP3/ROR- $\gamma$ t *in vitro*. Future studies will focus on the  
380 difference in cell components, such as cell wall proteins or sugars from these strains, to  
determine what combination of factors may be responsible for their immune modulating abilities.

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**Figures 1 a,b,c.** *In vitro* production of IL-2, IL-4, IL-6, IL-10, IL-12, IL-17, IFN- $\gamma$  and TGF- $\beta$ .  
640 Supernatants of □48 and ■72 co-cultures of pooled buffy coat-derived PBMC with live, GIT simulated and heat killed *Lb. acidophilus* LAVRI-A1, *Lb. rhamnosus* GG, EPS-producing *S. thermophilus* St1275, *B. longum* BL536, *B. lactis* B94 or *E. coli* TG1 or PBMC in medium alone were collected. The concentration of cytokines was subsequently determined using ELISA kits. Data are expressed as the mean cytokine response minus controls (pg/mL) of each treatment  
645 from triplicate wells, plus or minus the standard error of the mean (SEM).

**Figures 2 a,b.** *In vitro* production of IL-10 and IL-17. Supernatants of □48 and ■72 h co-cultures of CRL9850 or cord blood-derived PBMCs with live, GIT simulated or killed *Lb. acidophilus* LAVRI-A1, *Lb. rhamnosus* GG, EPS-producing *S. thermophilus* St1275, *B. longum*  
650 BL536, *B. lactis* B94 or *E. coli* TG1 or PBMC in medium alone were collected. The concentration of cytokines was subsequently determined using ELISA kits. Data are expressed as the mean cytokine response minus controls (pg/mL) of each treatment from triplicate wells, plus or minus the standard error of the mean (SEM).

**Figure 3.** Expression of activation marker CD25 by lymphocytes in response to selected bacteria. PBMCs were cultured with *Lb. acidophilus* LAVRI-A1, *Lb. rhamnosus* GG, EPS-producing *S. thermophilus* St1275, *B. longum* BL536, *B. lactis* B94 or *E. coli* TG1 for 72 h and evaluated on the expression of CD25 on T lymphocytes after 72 h of co-culture. Plots were gated on CD3. One representative experiment is shown of three different donors and from six strains (live and  
660 killed) used in these experiments.

**Figure 4.** PBMCs were co-cultured with Live or heat killed *Lb. acidophilus* LAVRI-A1, *Lb. rhamnosus* GG, EPS-producing *S. thermophilus* St1275, *B. longum* BL536, *B. lactis* B94 or *E. coli* TG1 in a ratio that does not induce apoptosis, LPS or cells alone as control. The percentage  
665 of induced CD25+FoxP3+ cells [A], and the induction of ROR- $\gamma$ t expressing Th17 cells [B], were assessed intracellularly by FACSCalibur after 96 h of co-culture. (b) Representative FACS plots for cultures described in (a); (d) Representative FACS plots for cultures described in (c).

Data are expressed as means plus or minus standard error of the mean (SEM) of three independent experiments.

**Table 1a: Cytokine levels of cultured PBMCs in response to live LAB**

Donor	Strains	Cytokine production upon bacterial treatment(pg/mL)															
		IL-2		IL-4		IL-6		IL-10		IL-12		IL-17		IFN-g		TGF-β	
		48 h	72 h	48 h	72 h	48 h	72 h	48 h	72 h	48 h	72 h	48 h	72 h	48 h	72 h	48 h	72 h
1	La	4.1±1.7	ND	5.4±1.5	10.7±2.4	41±13.6	20.6±5.2	14.4±3.1	15.6±4.4	ND	56.8±15.7	32±5.1	325±41.3	41.8±10.6	ND	2264±564	7400±1264
	LGG	22.4±4.8	67.1±20.2	1.4±0.4	ND	59±18.8	182±41	23.2±2.9	34.4±8.5	ND	ND	68±12.3	116±20	18.4±1.4	34.5±5.3	1246±287	2350±525
	Bl	13.5±3.1	374±92	47.1±12.1	5.4±1.7	19±8.3	213±33	6.4±1.3	24±8.3	135±32	29.3±3.5	ND	18±1.5	20.9±5.2	60.4±5.8	1478±324	4300±423
	B94	18.2±2.6	32.9±8.1	25.7±8.2	23±6.1	218±45.3	503±128	22.8±3.4	68.4±12.5	6.25±1.2	32.5±4.9	34±6.1	26±3.6	29.7±4.8	35.6±2.6	75±4.8	250±57
	St	48.2±12.6	37.6±7.5	63.6±21.2	60.7±12.5	332±63.8	2314±225	46.8±14.3	87.2±20.6	ND	ND	1497±312	2200±234	7.6±1.6	86.9±9.8	910±154	9750±2460
	Ec	420±125	338±78	ND	ND	220±52	44.3±13.8	ND	ND	ND	ND	12±0.8	18±1.4	ND	ND	850±206	550±57
2	La	ND	ND	ND	10.4±2.9	122±33	131±26	ND	ND	613±112	1170±286	8±1.5	ND	15.1±3.3	58.5±6.1	205±41	5050±1263
	LGG	123±29	422±102	1.4±0.6	7.9±2.3	147±28	972±204	ND	ND	158±32	1152±254	ND	ND	24.7±8.1	37.6±4.7	185±14	215±42
	Bl	25.9±5.6	ND	39±11	ND	129±24	769±198	ND	32.4±6.9	584±82	1267±376	25±4.3	36±2.8	24.6±3.8	66.6±5.2	4865±1204	500±62
	B94	8.8±2.3	ND	11.4±2.9	7.1±1.9	145±31	903±226	23.8±6.9	187±51	384±54	1000±154	8±2.6	42±12.5	36.2±6.2	49.3±6.7	4555±671	1250±429
	St	9.4±1.8	20.6±6.3	15.4±3.8	ND	128±18	1682±371	73.2±21.3	18.4±4.8	ND	ND	80±11.4	74±6.8	19.2±1.7	50±11.2	415±41.3	18350±5410
	Ec	1128±35	338±102	26±4	ND	520±108	676±91	ND	ND	925±175	415±57	ND	ND	131±22	ND	300±44	24300±4630
3	La	ND	22.2±4.7	0.7±0.3	1.8±0.5	21.7±4.9	12.7±2.9	6.8±1.7	23.6±6.6	4±0.4	50±10.5	108±14	76±12.1	11±2.1	12±3.6	ND	60±5.6
	LGG	1.8±0.4	ND	5.3±1.8	10±2.5	23.3±5.7	8±3.1	13.6±2.8	735±183	21.8±3.7	16.2±2.4	20±2.6	40±5.3	8±1.1	31.2±10.8	40.5±13.5	425±65
	Bl	5.9±1.2	2.9±1.1	1±0.3	2±0.4	2.7±0.5	2.3±0.4	12.4±2.9	541±144	8.1±1.7	40.6±3.2	ND	ND	25±3.8	ND	160±28	165±26
	B94	25.9±12.5	ND	1.8±0.6	2±0.6	10±1.4	46.7±11.5	7.6±1.1	22.4±9.8	28.7±4.1	5.6±1.4	410±52	86±12	20.5±5.6	28.4±9.2	70±22	125±33
	St	2.4±1.1	ND	1.4±0.5	5±1.1	38±11.6	13.7±2.9	4.4±0.8	13.2±2.4	10±1.6	6.8±1.1	28±4.8	60±3.8	24.6±2.6	29.8±7.6	80±19.5	125±25
	Ec	34.4±11.4	42.2±13.3	ND	ND	12.7±1.4	28.5±6.8	8.3±1.5	4.1±1.1	37.5±12.5	22.5±7.3	38±2.6	318±44	40±2.9	4±1.3	ND	ND



**Table 1b: Cytokine levels of cultured PBMCs in response to GIT LAB**

Donor	Strains	Cytokine production upon bacterial treatment(pg/mL)															
		IL-2		IL-4		IL-6		IL-10		IL-12		IL-17		IFN-g		TGF-β	
		48 h	72 h	48 h	72 h	48 h	72 h	48 h	72 h	48 h	72 h	48 h	72 h	48 h	72 h	48 h	72 h
1	La	6.6±1.2	30±5.3	ND	ND	52.8±13.2	124±22	90±12.6	78±21.4	57.5±13.6	525±105	8±2.4	152±24	2.1±0.5	12.8±2.6	2180±342	9400±2546
	LGG	7.5±2.8	16.6±4.2	ND	ND	57.1±14.5	104±15	52.5±8.8	32±4.5	305±102	227±62	440±128	218±20	3.4±0.9	9.6±3.2	3850±452	2400±638
	Bl	8.8±1.2	6.4±1.8	10±2	ND	28.5±5.9	ND	62.5±9.7	41±6.8	242±44	570±83	36±2.9	ND	ND	ND	350±41	3800±465
	B94	ND	ND	ND	ND	ND	ND	ND	ND	87.5±12.6	43.2±10.2	ND	ND	2.4±1.1	4.7±0.6	348±33	410±75
	St	18.8±5.2	197±33	ND	ND	1045±225	520±132	222±45	800±156	ND	ND	504±46	90±15.6	14.2±3.5	41.8±12.2	1100±204	6550±524
	Ec	ND	ND	91.4±21.5	ND	17.1±3.4	288±54	12±2.2	18±4.8	42.5±6.4	30±5.4	24±4.5	48±12.2	18.7±4.8	87.2±14.6	5450±1425	1300±156
2	La	28.8±12.3	38.8±6.3	ND	ND	2542±643	2118±345	218±56	670±136	22.5±6.5	8.4±2.3	ND	ND	5.7±2.6	15.4±3.4	700±133	1050±245
	LGG	12.2±2.6	24.1±12.5	ND	ND	901±124	843±106	27.5±6.4	100±22	132±25	53.2±15.4	ND	ND	14.2±3.8	27.1±6.2	350±62	500±85
	Bl	28.8±11.2	26.4±5.8	ND	62.8±21.1	768±129	478±56	8.5±1.4	87.5±32.5	225±35	30±5.9	ND	ND	7.8±1.9	11.4±2.4	128±24	250±36
	B94	508±136	412±69	ND	611±124	2504±356	1582±314	472±78	727±212	ND	ND	52±11.6	42±5.6	20±4.6	14.2±3.1	650±34	700±152
	St	11.1±1.6	8.6±2.6	15.7±2.6	45.7±12.6	905±145	818±146	13.8±3.6	107±17	1.8±0.8	3.4±0.8	270±25	226±53	22.8±4.6	70±10.6	4100±654	7350±1545
	Ec	ND	ND	ND	17.1±3.5	1558±416	957±178	ND	ND	16.4±3.3	45±5.6	32±8.4	ND	ND	21.4±3.6	3150±224	1300±362
3	La	25.5±5.9	13.2±3.6	ND	ND	11.4±4.2	17.1±3.6	5±1.2	9.3±2.8	ND	ND	22±4.2	48±6.7	2.3±0.7	1.8±0.4	280±25	1350±254
	LGG	129±41	434±125	ND	ND	7.1±2.4	42.8±12.5	3.2±0.5	ND	ND	ND	8±1.4	4.4±1.2	4.7±1.3	6.3±1.4	1475±453	3250±245
	Bl	7.7±2.3	3.6±0.9	ND	ND	21.4±3.6	19.8±3.4	1.8±0.4	2.4±0.6	1000±212	284±44	ND	ND	5.1±1.2	2.9±0.6	725±45	1584±128
	B94	166±29	42.8±11.3	142±35	394±67	50±8.5	78.5±19.4	ND	ND	1107±158	382±51	ND	ND	6.7±2.3	21.4±3.9	1450±275	5200±1526
	St	11.1±2.5	4.4±1.1	44.2±11.2	84.3±15.3	58.5±12.6	745±135	170±32.4	187±22	1217±324	432±58	128±26.3	106±25.6	4.4±1.5	12.3±2.4	350±34	7250±2547
	Ec	90±32.5	475±105	12.9±2.5	70±21	11.4±4.1	37.1±6.9	ND	ND	ND	ND	ND	ND	ND	ND	423±68	284±62

Gastrointestinal tract (GIT)

**Table 1c: Cytokine levels of cultured PBMCs in response to heat killed LAB**

Donor	Strains	Cytokine production upon bacterial treatment(pg/mL)															
		IL-2		IL-4		IL-6		IL-10		IL-12		IL-17		IFN-g		TGF- $\beta$	
		48 h	72 h	48 h	72 h	48 h	72 h	48 h	72 h	48 h	72 h	48 h	72 h	48 h	72 h	48 h	72 h
1	La	178 $\pm$ 32	84.4 $\pm$ 21.4	ND	ND	20 $\pm$ 5.6	132 $\pm$ 24	7.1 $\pm$ 1.6	84.2 $\pm$ 21.3	12.1 $\pm$ 2.6	13.4 $\pm$ 2.3	256 $\pm$ 54	142 $\pm$ 32	4.1 $\pm$ 1.5	10 $\pm$ 4.2	1200 $\pm$ 300	1600 $\pm$ 288
	LGG	3.8 $\pm$ 0.5	7.1 $\pm$ 1.6	51.3 $\pm$ 8.5	102 $\pm$ 16	37.1 $\pm$ 8.6	412 $\pm$ 114	2.8 $\pm$ 0.8	9.6 $\pm$ 2.4	255 $\pm$ 33	121 $\pm$ 23	128 $\pm$ 32.5	74 $\pm$ 14	40 $\pm$ 11.8	12.8 $\pm$ 3.6	3100 $\pm$ 1510	4100 $\pm$ 1885
	BI	ND	ND	351 $\pm$ 52	182 $\pm$ 33	135 $\pm$ 32.5	197 $\pm$ 35.6	37.1 $\pm$ 12.4	22.8 $\pm$ 4.4	12.5 $\pm$ 3.5	30 $\pm$ 4.8	12.9 $\pm$ 2.6	52 $\pm$ 12	110 $\pm$ 24.7	63.2 $\pm$ 16.4	4600 $\pm$ 1652	1300 $\pm$ 365
	B94	ND	ND	13.2 $\pm$ 3.4	4.8 $\pm$ 1.2	95.7 $\pm$ 24.6	127 $\pm$ 22.5	70 $\pm$ 10.5	101 $\pm$ 15.6	15 $\pm$ 2.8	26.5 $\pm$ 6.7	112 $\pm$ 24	42 $\pm$ 8.5	60 $\pm$ 8.9	41.8 $\pm$ 9.8	4150 $\pm$ 2150	800 $\pm$ 210
	St	6.6 $\pm$ 1.5	38.8 $\pm$ 6.8	54.2 $\pm$ 11.4	5.7 $\pm$ 1.6	14.2 $\pm$ 3.6	32.8 $\pm$ 6.8	31.4 $\pm$ 9.5	14.7 $\pm$ 3.8	27.5 $\pm$ 4.5	11.4 $\pm$ 4.2	2.8 $\pm$ 0.6	1.3 $\pm$ 0.4	130 $\pm$ 24	12.4 $\pm$ 3.7	1250 $\pm$ 315	2450 $\pm$ 158
	Ec	17.7 $\pm$ 3.6	4.2 $\pm$ 0.8	27.1 $\pm$ 4.6	52.8 $\pm$ 11.4	28.5 $\pm$ 4.5	14.7 $\pm$ 3.4	94.2 $\pm$ 13.4	308 $\pm$ 42	33.7 $\pm$ 8.4	51.9 $\pm$ 6.3	ND	ND	55.7 $\pm$ 12.2	14.2 $\pm$ 3.9	1600 $\pm$ 285	5400 $\pm$ 1425
2	La	210 $\pm$ 35	82.3 $\pm$ 21.2	3.4 $\pm$ 0.8	1.9 $\pm$ 0.6	204 $\pm$ 36.8	192 $\pm$ 32.2	11.4 $\pm$ 4.5	40 $\pm$ 8	4.3 $\pm$ 1.2	9.6 $\pm$ 1.3	182 $\pm$ 28	66 $\pm$ 6.6	118 $\pm$ 28.8	188 $\pm$ 36	650 $\pm$ 214	2050 $\pm$ 380
	LGG	171 $\pm$ 25	114 $\pm$ 22	24.2 $\pm$ 3.4	11.5 $\pm$ 2.6	55.7 $\pm$ 12.5	37.1 $\pm$ 6.6	ND	ND	95 $\pm$ 22.1	47.3 $\pm$ 6.4	10 $\pm$ 4	106 $\pm$ 22	14.3 $\pm$ 2.9	97.1 $\pm$ 24.6	450 $\pm$ 113	245 $\pm$ 82
	BI	ND	ND	12.8 $\pm$ 4.4	208 $\pm$ 35	ND	ND	431 $\pm$ 108	554 $\pm$ 107	35 $\pm$ 5.6	41.8 $\pm$ 10.4	84 $\pm$ 23.6	24 $\pm$ 4.6	118 $\pm$ 24	38.5 $\pm$ 11.3	750 $\pm$ 274	6050 $\pm$ 1450
	B94	ND	3.7 $\pm$ 0.5	ND	ND	127 $\pm$ 18.6	235 $\pm$ 35	35.7 $\pm$ 6.8	23.4 $\pm$ 6.3	4.3 $\pm$ 0.8	ND	30 $\pm$ 6.5	14.6 $\pm$ 3.8	8.5 $\pm$ 1.7	124 $\pm$ 24	2700 $\pm$ 875	1300 $\pm$ 278
	St	5.8 $\pm$ 1.7	14.1 $\pm$ 2.8	ND	ND	54.2 $\pm$ 13.6	118 $\pm$ 12	734 $\pm$ 124	1214 $\pm$ 312	5 $\pm$ 1.2	3.8 $\pm$ 0.8	74 $\pm$ 12.5	20 $\pm$ 3.3	165 $\pm$ 42	32.3 $\pm$ 7.8	1400 $\pm$ 215	625 $\pm$ 85
	Ec	4.8 $\pm$ 0.6	ND	27.1 $\pm$ 5.2	52.8 $\pm$ 8.5	28.5 $\pm$ 8.5	42.8 $\pm$ 8.4	258 $\pm$ 52	688 $\pm$ 198	ND	ND	216 $\pm$ 46	289 $\pm$ 42	48.5 $\pm$ 12.3	192 $\pm$ 46	8500 $\pm$ 2250	4300 $\pm$ 1250
3	La	27.7 $\pm$ 6.4	15.5 $\pm$ 4.5	27.1 $\pm$ 5.5	37.4 $\pm$ 6.4	42.8 $\pm$ 11.5	38.5 $\pm$ 4.8	13.3 $\pm$ 4.3	75 $\pm$ 24.7	60 $\pm$ 7.5	115 $\pm$ 21.5	40 $\pm$ 11.2	518 $\pm$ 78	121 $\pm$ 18	56.7 $\pm$ 13.3	5850 $\pm$ 1815	3050 $\pm$ 815
	LGG	18.8 $\pm$ 3.5	8.3 $\pm$ 3.4	ND	ND	164 $\pm$ 32	7.4 $\pm$ 2.6	96.6 $\pm$ 14.6	171 $\pm$ 52	67.5 $\pm$ 15.5	52.8 $\pm$ 8.8	60 $\pm$ 15.4	1140 $\pm$ 254	287 $\pm$ 52	132 $\pm$ 28	1250 $\pm$ 235	2550 $\pm$ 520
	BI	45.5 $\pm$ 5.9	77.7 $\pm$ 15.4	55.7 $\pm$ 16.3	231 $\pm$ 46	52.8 $\pm$ 12.6	138 $\pm$ 25	176 $\pm$ 28	203 $\pm$ 45	670 $\pm$ 125	710 $\pm$ 124	78 $\pm$ 13	236 $\pm$ 44	252 $\pm$ 46	101 $\pm$ 21	1200 $\pm$ 157	5250 $\pm$ 1728
	B94	67.7 $\pm$ 9.5	121 $\pm$ 32	25.7 $\pm$ 7.5	43.2 $\pm$ 9.6	12.4 $\pm$ 2.6	35.7 $\pm$ 5.6	25 $\pm$ 6.5	32 $\pm$ 6	35 $\pm$ 4.6	327 $\pm$ 52	548 $\pm$ 42	274 $\pm$ 38	83.4 $\pm$ 17.4	177 $\pm$ 35	2250 $\pm$ 385	8450 $\pm$ 2415
	St	34.2 $\pm$ 4.3	53.3 $\pm$ 11.5	51.4 $\pm$ 11.3	8.6 $\pm$ 2.4	170 $\pm$ 25	112 $\pm$ 22	343 $\pm$ 45	391 $\pm$ 45	ND	ND	12 $\pm$ 4	20 $\pm$ 4.2	288 $\pm$ 62	34.2 $\pm$ 6.8	1300 $\pm$ 220	2450 $\pm$ 425
	Ec	15.5 $\pm$ 2.9	23.3 $\pm$ 6.5	ND	ND	55.7 $\pm$ 12.5	84.2 $\pm$ 21.2	23.3 $\pm$ 6.5	58.8 $\pm$ 18.2	305 $\pm$ 42	128 $\pm$ 29	70 $\pm$ 11.6	98 $\pm$ 21.6	14.1 $\pm$ 3.7	32.8 $\pm$ 11.2	4100 $\pm$ 1100	1600 $\pm$ 265

Values are means  $\pm$  standard error of the mean (Three healthy donors). Data differ significantly ( $P < 0.05$ ). *Lactobacillus* (Lb.) *acidophilus* LAVRI-A1; *Lb. rhamnosus* GG (LGG); *Bifidobacterium* (B.) *lactis* B94; *B. longum* BL536; EPS producing *Streptococcus* (S.) *thermophilus* St1275; *Escherichia* (E.) *coli*; TG1; Interleukin (IL); Interferon (IFN); transforming growth factor (TGF).

**Table 2: Enumeration of bacteria after 18 h incubation**

Treatment	<u>Cell count (CFU)/mL</u>											
	LAVRI-A1		LGG		BI536		B94		St1275		E. coli	
	0 h	18 h	0 h	18 h	0 h	18 h	0 h	18 h	0 h	18 h	0 h	18 h
Live	7.47	9.06	7.00	9.29	7.31	8.74	7.10	8.65	7.10	8.77	6.89	8.56
GIT simulation	6.27	8.12	6.00	8.43	6.10	8.53	6.15	8.39	6.00	8.02	6.00	8.27
Killed	0	0	0	0	0	0	0	0	0	0	0	0
SEM	0.24											

Results presented as a mean (n=3) ± pooled standard error of the mean (0.243). *Lactobacillus* (Lb.) *acidophilus* LAVRI-A1; *Lb. rhamnosus* GG (LGG); *Bifidobacterium* (B.) *lactis* B94; *B. longum* BL536; EPS producing *Streptococcus* (S.) *thermophilus* St1275; *Escherichia* (E.) *coli*.

Figure 1a. Live bacteria induction of PBMC – pro- and anti-inflammatory cytokines

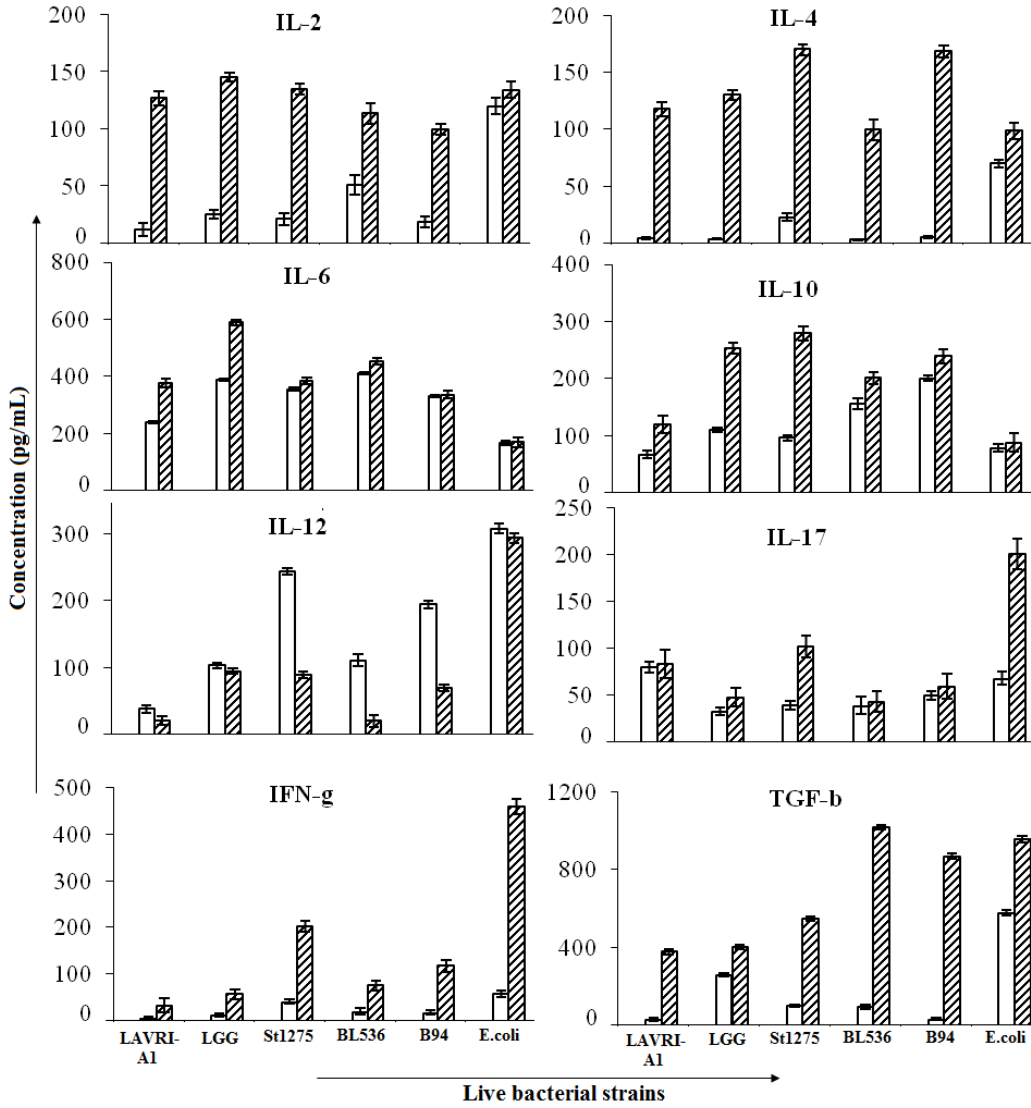


Figure: 1b GIT simulated bacteria induction of PBMC – pro- and anti-inflammatory cytokines

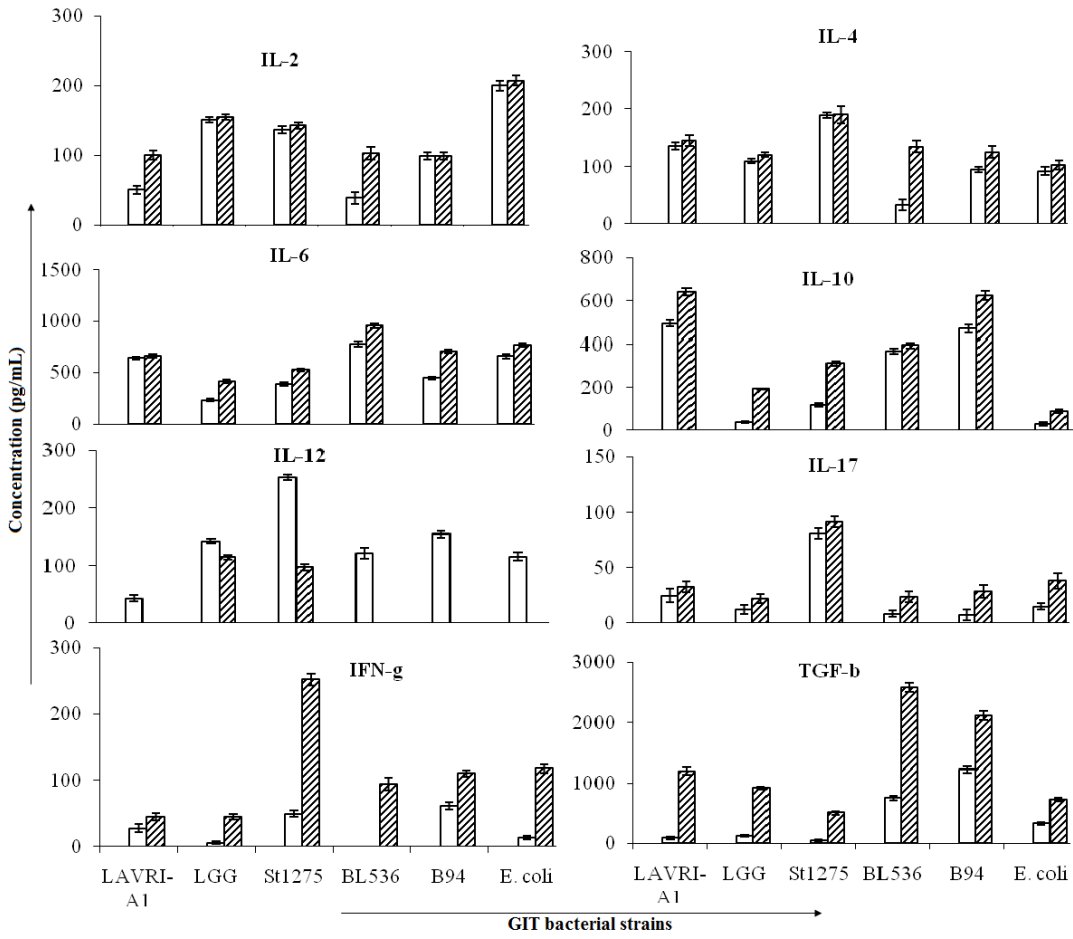


Figure 1c Killed bacteria induction of PBMC – pro- and anti-inflammatory cytokines

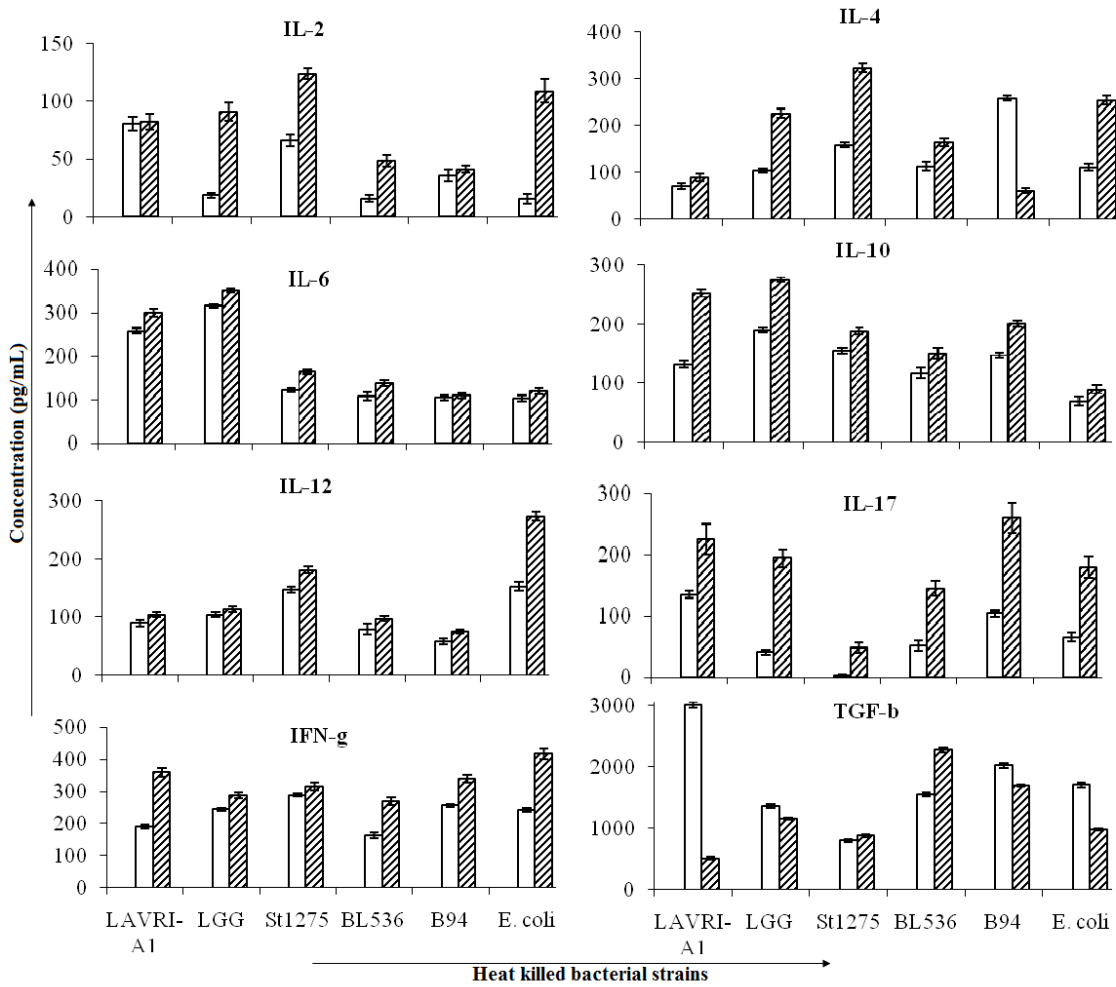


Figure 2a Production of IL-10

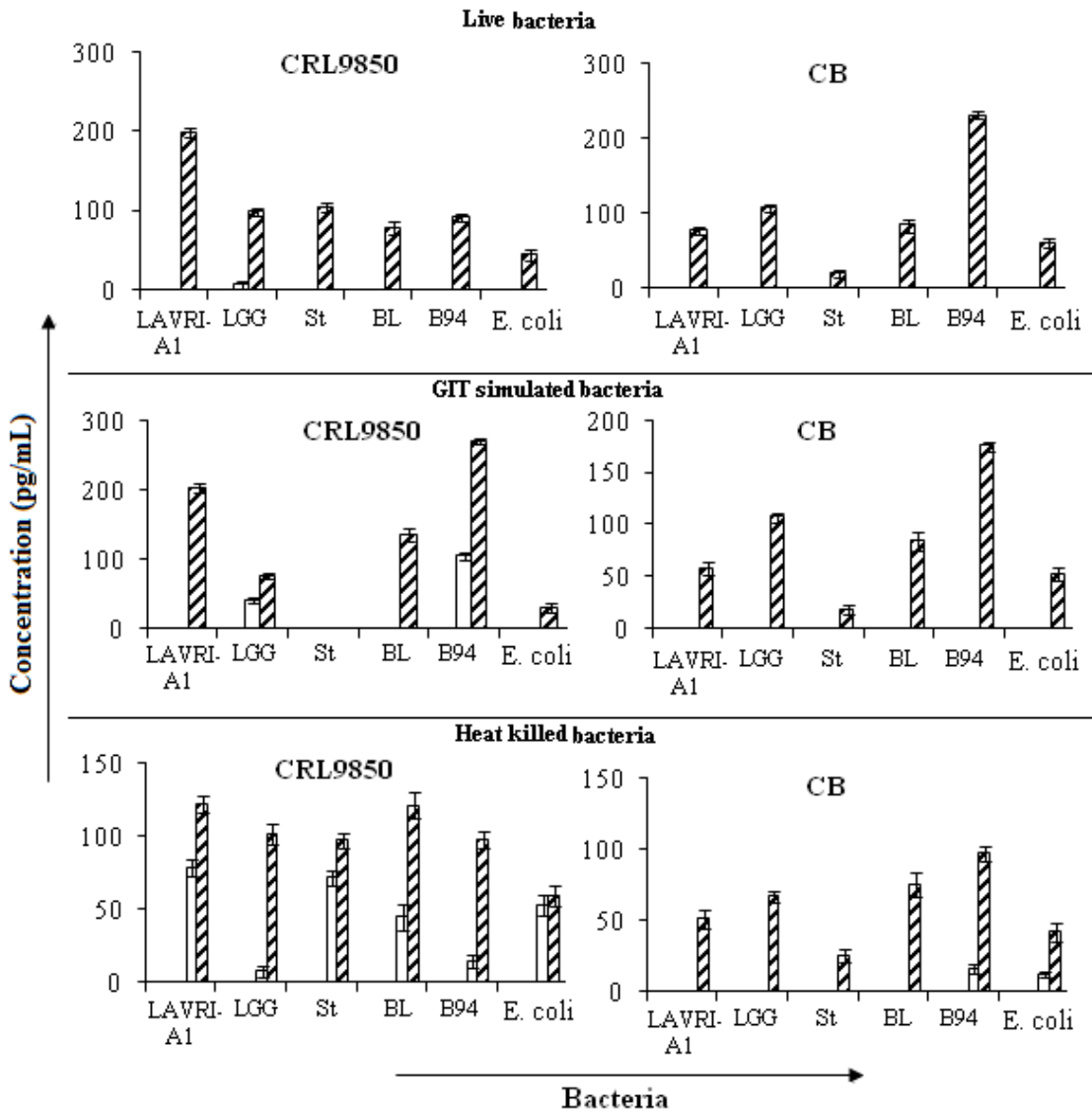


Figure 2b. Production of IL-17

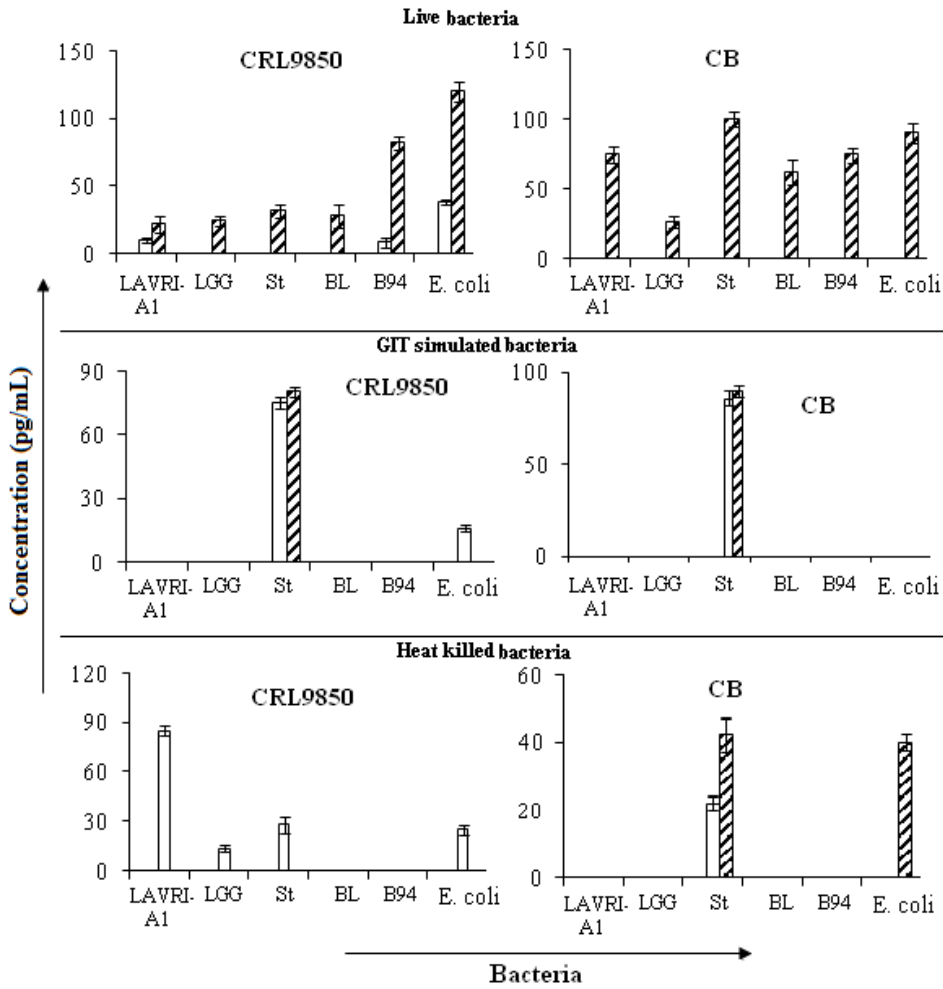




Figure 3:

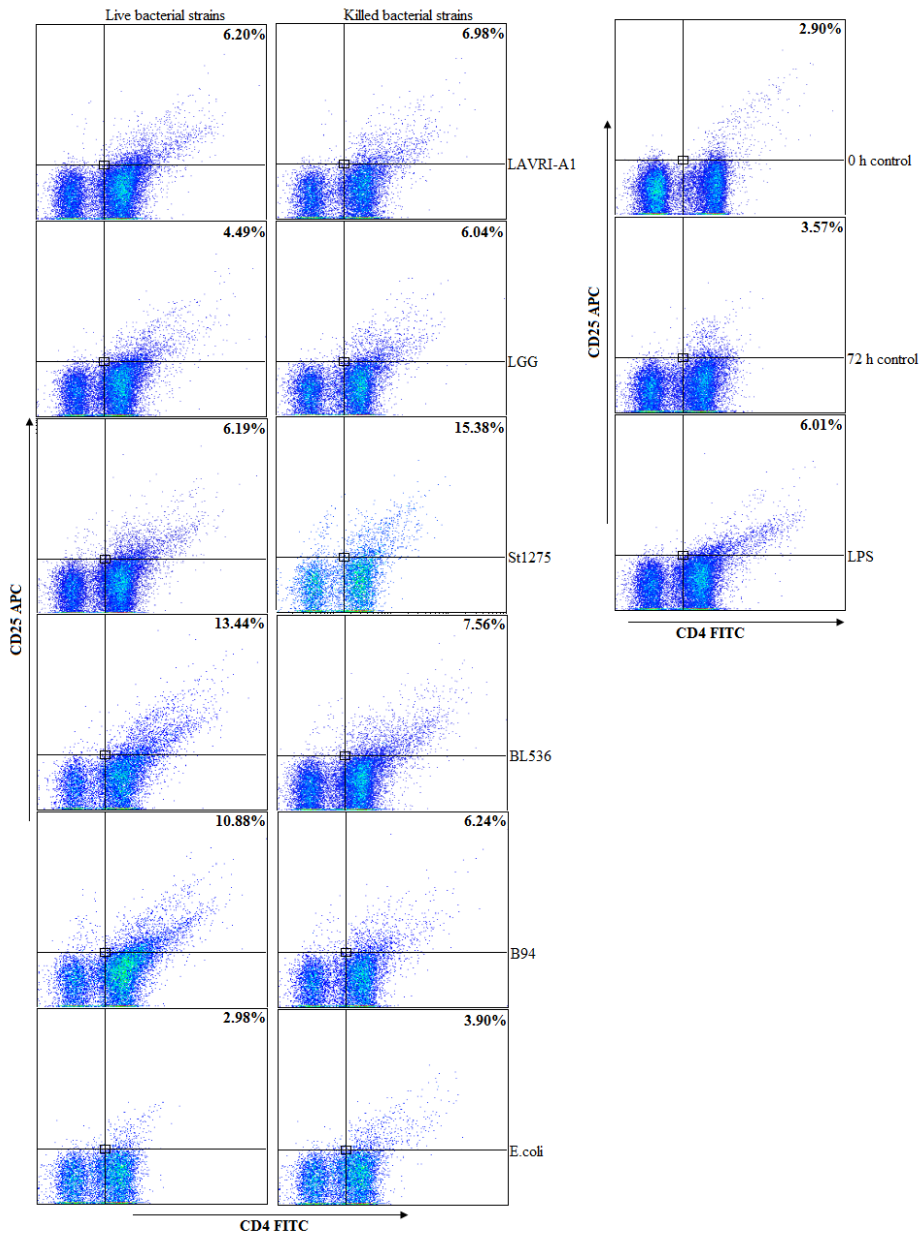
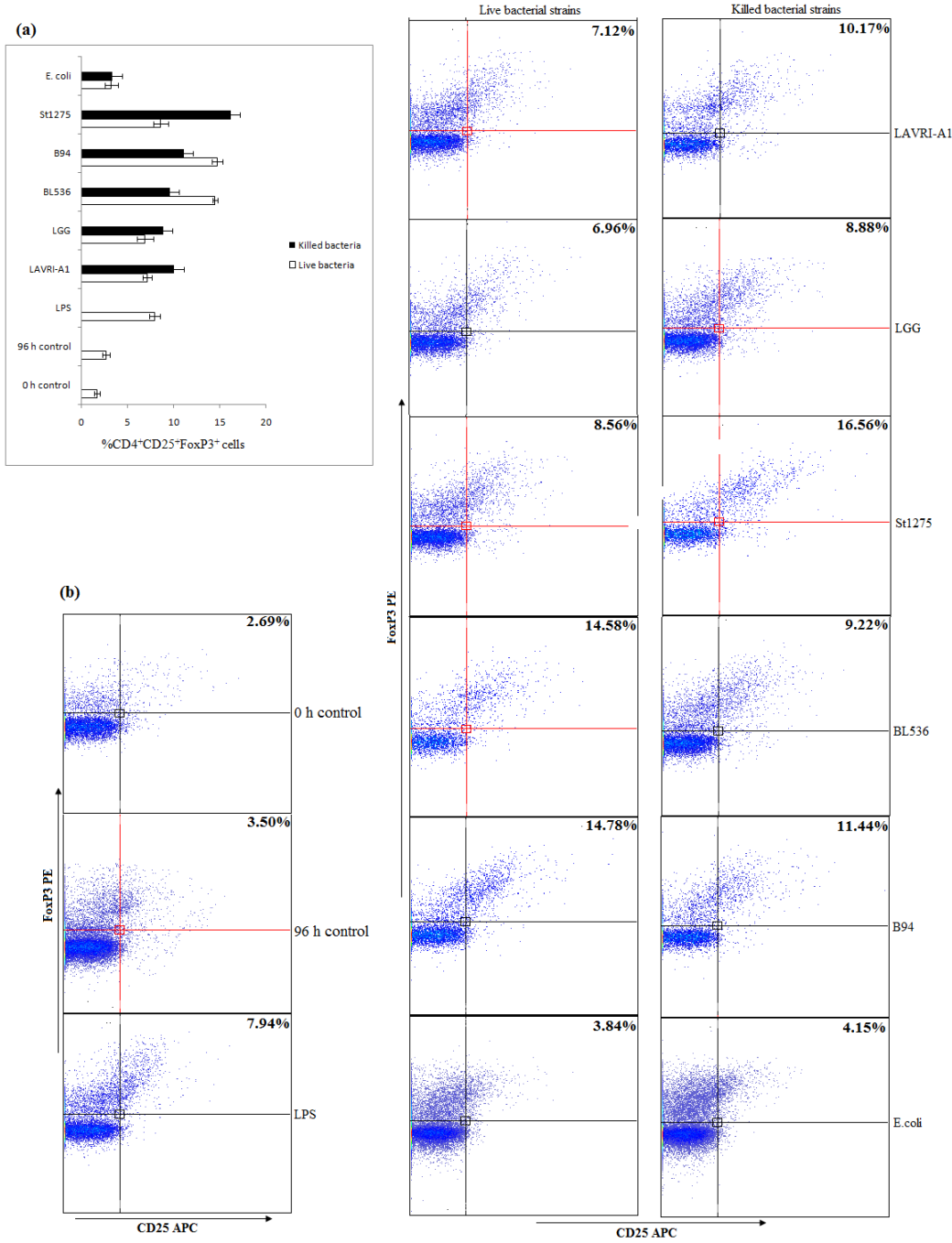


Figure 4

[A] a,b



[B] c,d

