*Cytokine profile and induction of T helper type 17 and regulatory T cells by human peripheral mononuclear cells after microbial exposure*

This is the Accepted version of the following publication

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

O. Donkor [a*], M. Ravikumar [a], O. Proudfoot[b], SL. Day[b], V. Apostolopoulos[b], T. Vasiljevic [a], S. Nutt[c] and H. Gill[d]

[a]School of Biomedical and Health Sciences, Victoria University, Werribee Campus, P.O. Box 14428, Melbourne, Vic 8001, Australia.
[b]Burnet Institute, Centre for Immunology, Melbourne, Vic, 3004, Australia.
[c]The Walter and Eliza Hall Institute of Medical Research, Parkville, Vic 3050, Australia
[d]Department of Primary Industries, Werribee, Vic 3030, Australia.

*Corresponding author: Dr O. N. Donkor; Tel: (+61-3)-9919-8059, Fax: (+61-3)-99198284
 e-mail: Osaana.Donkor@vu.edu.au

*5*

*10*

*15*

*20*

*25*

*30*

*1*

**Abstract**

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, *Bifidobacteriun longum* BL536, *Bifidobacteriun lactis* B94 and *Escherichia coli* TG1 strains. Cell production of a panel of pro- and anti-inflammatory 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 significant concentrations of TGF-β, 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 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 potently inducing differentiation of Th17 and Treg cells.

**Clinical and Experimental Immunology**

**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-α and IFN-γ, 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$^+$ 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$^+$ 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 *Bifidobacterium lactis* Bb12 and *Lb. rhamnosus* GG, suggesting the induction of regulatory cells

*85*    by these strains [23]. The important discovery that TGF-β 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-β 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*    β 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.

**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 $10^7$ 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, $10^7$ 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 $10^7$ CFU/vial in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 1% L-glutamine (Sigma).

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).

125

*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]

130 was used with some modifications as described. To simulate bacterial digestion in the stomach, 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$_3$, 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 (untreated), GIT and killed) was 10-fold serially diluted and plated onto tryptic soy agar (*E. coli*),

145 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 Hessle 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 $NH_4Cl$, 0.01 M $KHCO_3$ and 10 µM EDTA $Na_2.2H_2O$) 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 $10^6$ cells/well and incubated at 5% $CO_2$, 37°C for 24 h prior to stimulation with bacteria as described by Amrouche et al. [31]. Briefly, $10^6$ freshly-prepared viable (live or GIT) or equivalent (~$10^6$ CFU/mL) heat-killed bacteria were added per $10^6$ cells and co-cultured for 72 h

175    at 5% $CO_2$, 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.

180

*Cell staining and Flow Cytometry analysis*

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

or media alone, in a 24-well plate at 37°C in 5% $CO_2$ 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 $10^6$ cells/mL, and surface marker staining was performed using fluorescein isothiocynate (FITC) -labeled anti-human CD4, allophycocyanin -labeled anti-human 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 Gatelogic 3.07 software (Inivai, Australia). Absolute numbers of Treg cells and Th17 cells were calculated as a percentage of the 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 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 $p < 0.05$.

**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 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 significantly higher ($p<0.05$) concentrations (100 – 8800 pg/mL) of cytokines compared to cord

220  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-β, 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 contrastLAVRI-A1, B94, BL536, ST1275 and LGG

were found to stimulate high levels of IL-10 (Fig. 1b). From CRL9850 and cord blood derived-PBMCsonly LAVRI-A1, LGG, Bl536 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-γt *expression in PBMCs by live/killed selected bacteria strains*

PBMC incubation with LAB resulted in enhanced expression of CD25 on CD4$^+$ 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$^+$CD25$^+$FoxP3$^+$) 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$^+$ CD4$^+$ 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-γt were subsequently used. The proportion of Th17 cells (CD3$^+$CD4$^+$CCR6$^+$ROR-

270     γt$^+$) 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-γ 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-γ 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-γ production in T cells and natural killer cells, which accelerates the development of naive CD4$^+$ T cells into Th1-type cells [51]. Therefore, IL-12 is a key immunoregulator favouring Th1-type responses. However IFN-γ in turn induces IL-12 production, which can cause a positive feedback loop of IFN-γ 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-β 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-β, 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-β1 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-γ, and TNF-α production. The severe and uncontrolled inflammatory reactions observed in the TGF-β1 knockout mouse attests to the physiologic role of TGF-β 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.

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 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,* bifidobateria 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 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-β has been shown to be involved in both Treg and Th17 development. Animal models have demonstrated that at high levels of TGF-β, Foxp3 expression is upregulated and Treg differentiation is induced, whereas at low levels of TGF-β, IL-6 and IL-21 synergize to promote the differentiation of Th17 cells [88]. The identification of the transcription factor ROR-γt by intracellular and CCR6 extracellular staining confirms the differentiation of Th17 cells in the current experiment. Th17 cells induce a range of pro-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 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$^+$ Treg induction in the co-cultures still remains unclear. TGF-β 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-β were among the various cytokines released. Furthermore, we have shown that production of cytokines and induction of ROR-γt/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+ regulatory cells *in vitro*, independent of the IL-10 production. The

*375* overall extent of induction of FoxP3+ (Treg) and ROR-γt+ (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-γt *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.

## Acknowledgements

*390*

## References:

1. McCartney AL, Wenzhi W, Tannock GW. Molecular analysis of the composition of the Bifidobacterial and Lactobacillus microflora of humans. *Appl Environ Microbiol* 1996;
*395* 62:4608–13.

2. Fooks LJ, Gibson GR. Probiotics as modulators of the gut flora. *British J Nutr* 2002; 88:S39– S49.

3. FAO/WHO. Guidelines for the evaluation of probiotics in food. Report of a joint FAO/WHO working group on drafting guidelines for the evaluation of probiotics in food. 2002; London
*400* ON, Canada.

4. Marteau P, Minekus M, Havenaar R, Huis in't veld JHJ. Survival of Lactic acid bacteria in a dynamic model of the stomach and small intestine: validation and the effects of bile. *J Dairy Sci* 1997; 80:1031–7.

5. Chin J, Turner B, Barchia I, Mullbacher A. Immune response to orally consumed antigens and probiotic bacteria. *Immunol Cell Biol* 2000; 78:55–6.

6. Pinsky MR. Sepsis: a pro- and anti-inflammatory disequilibrium syndrome. *Contrib Nephrol* 2001; 132: 354–6.

7. Gill HS. Best Practice and Research. *Clin Gastroenterol* 2003; 17:755-73.

8. Romagnani S. Immunologic influences on allergy and the TH1/TH2 balance. *J Allergy Clin Immunol* 2004; 113:395–400.

9. Shi Y, Wang H, Su Z et al. Differentiation Imbalance of Th1/ Th17 in Peripheral Blood Mononuclear Cells Might Contribute to Pathogenesis of Hashimoto's Thyroiditis. *Journal Scandinavian Journal of Immunology* 2010; 72:250–5.

10. Ivanov II, McKenzie BS, Zhou L et al. The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17 T helper cells. *Cell* 2006; 126:1121–33.

11. Chen Y, Langrish CL, McKenzie B et al. Anti-IL-23 therapy inhibits multiple inflammatory pathways and ameliorates autoimmune encephalomyelitis. *J Clin Invest* 2006;116:1317–26.

12. Park H, Li Z, Yang XO et al. A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. *Nat Immunol* 2005; 6:1133–41.

13. Bettelli E, Sullivan B, Szabo SJ et al. Loss of T-bet, but not STAT1, prevents the development of experimental autoimmune encephalomyelitis. *J Exp Med* 2004; 200:79–87.

14. Neurath MF, Weigmann B, Finotto S et al. The transcription factor T-bet regulates mucosal T cell activation in experimental colitis and Crohn's disease. *J Exp Med* 2002; 195:1129–43.

15. Lin W, Truong N, Grossman WJ et al. Allergic dysregulation and hyperimmunoglobulinemia E in FoxP3 mutant mice. *J Allergy Clin Immunol* 2005; 116:1106–15.

16. Bennett CL, Christie J, Ramsdell F et al. The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FoxP3. *Nat Genet* 2001; 27:20–1.

17. Walker MR, Kasprowicz DJ, Gersuk VH et al. Induction of FoxP3 and acquisition of T regulatory activity by stimulated human CD41CD25– T cells. *J Clin Invest* 2003; 112:1437–43.

18. Wang YH, Liu YJ. The IL-17 cytokine family and their role in allergic inflammation. *Curr Opinion Immunol* 2008; 20:697–702.

19. Seroogy C, Gern J. The role of T regulatory cells in asthma. *J Allergy Clin Immunol* 2005; 116:996–9.

20. Kalliomaki M, Salminen S, Poussa T, Isolauri E. Probiotics during the first 7 years of life: a cumulative risk reduction of eczema in a randomized, placebo-controlled trial. *J Allergy Clin Immunol* 2007; 119:1019–21.

21. Baharav E, Mor F, Halpern M, Weinberger A. Lactobacillus GG Bacteria Ameliorate Arthritis in Lewis Rats. *J Nutr* 2004; 134:1964–9.

22. Hatakka K, Martio J, Korpela M et al. Effects of probiotic therapy on the activity and activation of mild rheumatoid arthritis – a pilot study. *Scand J Rheumatol* 2003; 32:211–5.

23. Feleszko W, Jaworska J, Rha RD et al. Probiotic-induced suppression of allergic sensitization and airway inflammation is associated with an increase of T regulatory-dependent mechanisms in a murine model of asthma. *Clin Exp Allergy* 2007; 37:498–505.

24. Stockinger B, Veldhoen M. Differentiation and function of Th17 T cells. *Curr Opin Immuno* 2007; 19:281–6.

25. Bettelli E, Carrier Y, Gao W et al. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature* 2006; 441:235-8.

26. Mangan PR, Harrington LE, O'Quinn DB et al. Transforming growth factor-beta induces development of the T(H)17 lineage. *Nature* 2006; 441:231-4.

27. Wu CL, Chan MC, Chang GC et al. Aetiology and cytokine expression in patients requiring mechanical ventilation due to severe community-acquired pneumonia. *J Formos Med Assoc* 2006; 105:49–55.

28. Dinarello CA. Proinflammatory and anti-inflammatory cytokines as mediators in the pathogenesis of septic shock. *Chest* 1997; 112:321S–9S.

29. Opal SM, DePalo VA. Anti-inflammatory cytokines. *Chest* 2000; 117:1162–72.

30. Gill HS, Rutherfurd KJ. Viability and dose-response studies on the effects of the immunoenhancing lactic acid bacterium *Lactobacillus rhamnosus* in mice. *British J Nutr* 2001; 86:285–9.

31. Amrouche T, Boutinb Y, Prioulta G, Flissa I. Effects of bifidobacterial cytoplasm, cell wall and exopolysaccharide on mouse lymphocyte proliferation and cytokine production. *Int Dairy J* 2006; 16:70–80.

32. Hessle C, Andersson B, Wold AE. Gram-positive bacteria are potent inducers of monocytic interleukin-12 (IL-12) while Gram-negative bacteria preferentially stimulate IL-10 production. *Infect Immun* 2000; 68: 3581–6.

33. He F, Morita H, Ouwehand AC et al. Stimulation of the secretion of pro-inflammatory cytokines by *Bifidobacterium* strains. *Microbiol Immunol* 2002; 46:781–5.

34. Miettinen M, Matikainen S, Vuopio-Varkila J et al. Lactobacilli and streptococci induce interleukin-12 (IL-12), IL-18, and interferon gamma production in human peripheral blood mononuclear cells. *Infect Immun* 1998; 66:6058–62.

35. Dave RI, Shah NP. Evaluation of media for selective enumeration of *Streptococcus thermophilus*, *Lactobacillus delbrueckii* ssp. *bulgaricus*, *Lactobacillus acidophilus*, and bifidobacteria. *J Dairy Sci* 1996; *79*:1529-36.

36. Miquel E, Gómez JA, Alegría A, Barberá R, Farré R, Recio I. Identification of casein phosphopeptides after simulated gastrointestinal digestion by tandem mass spectrometry. *Europ Food Res Technol* 2006; 222:48–53.

37. Donkor ON, Henriksson A, Singh TK, Vasiljevic T, Shah NP. ACE-inhibitory activity of probiotic yoghurt. *Int Dairy J* 2007; 17:1321–31.

38. de Roock S, van Elk M, van Dijk MEA, Timmerman HM, Rijkers GT, Prakken BJ, Hoekstra MO, de Kleer I. M. Lactic acid bacteria differ in their ability to induce functional regulatory T cells in humans. *Clin Exp Allergy* 2009; 40:103–110.

39. SAS/STAT. Software: Changes and enhancements through release 6.11, SAS Inst. Inc. 1996 Cary, NC.

40. Niers LEM, Timmerman HM, Rijkers GT et al. Identification of strong interleukin-10 inducing lactic acid bacteria which down regulate T helper type 2 cytokines. *Clin Exp Allergy* 2005; 35:1481–9.

41. Breitling LP, Fendel R, Mordmueller B, Adegnika AA, Kremsner PG, Luty AJF. Cord Blood Dendritic Cell Subsets in African Newborns Exposed to Plasmodium falciparum In Utero. *Infect Immun* 2006; 74, 5725–9.

42. Malhotra IP, Mungai E, Muchiri J, Ouma S et al. Distinct Th1- and Th2-Type prenatal cytokine responses to Plasmodium falciparum erythrocyte invasion ligands. *Infect. Immun* 2005; 73:3462–70.

43. Hanna N, Hanna I, Hleb M, Wagner E et al. Gestational Age-Dependent Expression of IL-10 and Its Receptor in Human Placental Tissues and Isolated Cytotrophoblasts. *J Immun* 2000; 164: 5721–8.

44. Bennet WA, Lagoo-Deendayalan S, Whitworth NS, Stopple JA et al. First trimester human chorionic villi express both immunoregulatory and inflammatory cytokines: a role for interleukin-10 in regulating the cytokine network of pregnancy. *Am. J. Reprod. Immunol* 1999; 41:70.

45. Oozeer R, Leplingard A, Mater DDG et al. Survival of *Lactobacillus casei* in the Human digestive tract after consumption of fermented milk. *Appl Environ Microbiol* 2006; 5615–7.

46. Karimi K, Inman MD, Bienenstock J, Forsythe P. Lactobacillus reuteri–induced Regulatory T cells Protect against an Allergic Airway Response in Mice. *Am J Respir Crit Care Med* 2009, 179:186–193,

47. Hessle CC, Andersson B, Wold AE. Gram-positive and Gram-negative bacteria elicit different patterns of pro-inflammatory cytokines in human monocytes. *Cytokine* 2005; 30, 311-8.

48. Eastaff-Leung N, Mabarrack N, Barbour A, Cummins A, Barry S. FoxP3$^+$ Regulatory T Cells, Th17 Effector Cells, and Cytokine Environment in Inflammatory Bowel Disease. *J Clin Immunol* 2010; 30:80–8.

49. Minekus M, Marteau P, Havenaar R, Huis in't Veld JHJ. A multicompartmental dynamic computercontrolled computer controlled model simulating the stomach and small intestine. *Altern Lab Anim* 1995; 23:197-209.

50. Notermans S, Gallhoff G, Zwietering MH, Mead GC. The HACCP concept: specification of criteria using quantitative risk assessment. *Food Microbiol* 1995; 12:81-90.

51. Trinchieri G, Proinflammatory and immunoregulatory functions of interleukin-12. *Int. Rev. Immunol.* 1998: 16:365-96.

52. Matsuzaki T, Chin J. Modulating immune responses with probiotic bacteria. *Immunol Cell Biol* 2000; 78:67–73.

53. Kalliomaki M, Salminen S, Arvilommi H, Kero P, Koskinen P, Isolauri E. Probiotics in primary prevention of atopic disease: a randomized placebo-controlled trial. *Lancet* 2001; 357:1076-9.

54. Majamaa H, Isolauri E. Probiotics: a novel approach in the management of food allergy. *J. Allergy Clin. Immunol.* 1997; 99:179-85.

55. Malek TR. The main function of IL-2 is to promote the development of T regulatory cells. *J Leuk Biol* 2003; 74:961-5.

56. Liu Z, Jiu J, Liu S, Fa X, Li F and Du Y: Blockage of tumor necrosis factor prevents intestinal mucosal inflammation through down-regulation of interleukin-23 secretion. *J Autoimmun* 2007; 29:187-194.

57. Sartor RB. Microbial influences in inflammatory bowel diseases. *Gastroentero* 2008; 134:577-94.

58. Kolls JK, Linden A. Interleukin-17 family members and inflammation. *Immunity* 2004; 21: 467-76.

59. Bettelli E, Oukka M, Kuchroo VK. TH-17 cells in the circle of immunity and autoimmunity. *Nat Immunol* 2007; 8: 345-50.

60. Steinman L. A brief history of TH17, the first major revision in the TH1/TH2 hypothesis of T cell-mediated tissue damage. *Nat Med* 2007; 13: 139-45.

61. Meyaard L, Hovenkamp E, Otto SA, Miedma F. IL-12 induced IL-10 production by human T cells as a negative feedback for IL-12 induced immune responses. *J Immunol* 1996; 156, 2776-82.

62. Morita H, He F, Fuse T et al. Cytokine Production by the Murine Macrophage Cell Line J774.1 after Exposure to *Lactobacilli*. *Biosc Biotech Biochem* 2002; 66:1963-6.

63. Soichi T, Kinuta Y, Saito Y. *Bifidobacterium infantis* suppresses proinflammatory interleukin-17 production in murine splenocytes and dextran sodium sulfate-induced intestinal inflammation. *Int J Mole Med* 2008; 22: 181-5.

64. Lalani I, Bhoi K, Ahmed AF. Interleukin-10: biology, role in inflammation and autoimmunity. *Ann Allergy* 1997; 79:469–83.

65. Clarke CJP, Hales A, Hunt A, et al. IL-10 mediated suppression of TNF-alpha production is independent of its ability to inhibit NF-kB activity. *Eur J Immunol* 1998; 28:1719–26.

66. Litterio JJ, Roberts AB. TGF-b: a critical modulator of immune cell function. *Clin Immunol Immunopathol* 1997; 84:244–250.

67. Berg DJ, Kuhn R, Rajewsky K et al. Interleukin-10 is a central regulator of the response to LPS in murine models of endotoxic shock and the Shwartzman reaction but not endotoxin tolerance. *J Clin Investig* 1995; 96:2339–47.

68. Zoetendal EG, Rajilic-Stojanovic M, de Vos WM. High-throughput diversity and functionality analysis of the gastrointestinal tract microbiota. Gut 2008; 57:1605-15.

69. Maassen CB, Claassen E. Strain-dependent effects of probiotic lactobacilli on EAE autoimmunity. *Vaccine* 2008; 26:2056–7.

70. Gupta P, Andrew H, Kirschner BS, Guandalini S, Is *lactobacillus* GG helpful in children with Crohn's disease? Results of a preliminary, open-label study. *J. Pediatr Gastroenterol Nutr* 2000; 31, 453-7.

71. Klinman DM, Yi AK, Beaucage SL, Conover J, Krieg AM. CpG motifs present in bacteria DNA rapidly induce lymphocytes to secrete interleukin 6, interleukin 12, and interferon gamma. *Proc Natl Acad Sci* USA1996; 93:2879–83.

72. Lawrence C, Nauciel C. Production of interleukin-12 by murine macrophages in response to bacterial peptidoglycan. *Infect  Immun* 1998; 66:4947–9.

73. Cross ML, Ganner A, Teilab D, Fray LM.  Patterns of cytokine induction by gram-positive and gram-negative probiotic bacteria. FEMS Immunol Med Microbiol  2004; 42:173–80.

74. Macpherson AJ, Harris NL. Interactions between commensal intestinal bacteria and the immune system.  *Nat Rev Immunol* 2004; 4:478–85.

75. Kato I, Tanaka K, Yokokura T. Lactic acid bacterium potently induces the production of interleukin-12 and interferon-gamma by mouse splenocytes. *Int J Immunopharmacol* 1999 21:121–31.

76. De Waard R, Claassen E, Bokken GC, Buiting B, Garssen J, Vos JG. Enhanced immunological memory responses to Listeria monocytogenes in rodents, as measured by delayed-type hypersensitivity (DTH), adoptive transfer of DTH, and protective immunity, following *Lactobacillus casei* Shirota ingestion. *Clin Diagn Lab Immunol* 2003; 10:59–65.

77(5). Rodríguez C, Medici M, Mozzi F, Font de Valdez G. Therapeutic effect of *Streptococcus thermophilus* CRL1190-fermented milk on chronic gastritis. *World J Gastroenterol* 2010; 16:1622-30.

78. Hammes WP, Vogel RF. The genus *Lactobacillus* 1995. Blackie Academic & Professional, Glasgow.

79. He F, Morita H, Ouwehand AC. Bifidobacteria and lactobacilli exhibited different mitogenic activity on murine splenocytes. Int J Probio Prebio 2006; 1:77-82.

80. Nicaise P, Gleizes A, Forestier F, Quero A-M, Labarre C. Influence of intestinal bacterial flora on cytokine (IL-1, IL-6 and TNF-α) production by mouse peritoneal macrophages. *Europ Cyto Net* 1993; 4:133-8.

81. Kitazawa H, Tomioka Y, Matsumura K et al. Expression of mRNA encoding IFN alpha in macrophages stimulated with *Lactobacillus gasseri*. *FEMS Microbiol Letters* 1994; 120:315-21.

82. Rachmilewitz D, Katakura K, Karmeli F et al. Toll-like receptor 9 signaling mediates the anti-inflammatory effects of probiotics in murine experimental colitis. *Gastroenterol* 2004; 126:520–28.

83. Grangette C, Nutten S, Palumbo E et al. Enhanced anti-inflammatory capacity of a *Lactobacillus plantarum* mutant synthesizing modified teichoic acids. *Proceed Nat Acad Sc* 2005; 102:10321–6.

84. Michelsen KS, Aicher A, Mohaupt M, et al. The role of toll-like receptors (TLRs) in bacteria-induced maturation of murine dendritic cells (DCs). *J Biolo Chem* 2001; 276:25680–6.

85. Hopkins PA, Sriskandan S. Mammalian toll-like receptors: to immunity and beyond. *Clin Exp Immunol* 2005; 140:395–407.

86. Acosta-Rodriguez EV, Napolitani G, Lanzavecchia A, Sallusto F. Interleukins 1beta and 6 but not transforming growth factor-beta are essential for the differentiation of interleukin 17-producing human T helper cells. *Nat Immunol* 2007; 8:942–9.

87. Yang L, Anderson DE, Baecher-Allan C, Hastings WD, Bettelli E, Oukka M et al. IL-21 and TGF-beta are required for differentiation of human T(H)17 cells. *Nature* 2008; 454:350–2.

88. Zhou L, Lopes JE, Chong MM, Ivanov II, Min R, Victora GD et al. TGF-β-induced FoxP3 inhibits Th17 cell differentiation by antagonizing RORγt function. *Nature* 2008; 453:236–40.

89. LeibundGut-Landmann S, Gross O, Robinson MJ, Osorio F, Slack EC, Tsoni SV, et al. Syk- and CARD9-dependent coupling of innate immunity to the induction of T helper cells that produce interleukin 17. *Nat Immunol* 2007; 8:630–8.

90. Gaffen SL. An overview of IL-17 function and signaling. *Cytokine* 2008; 43:402–7.

91. Fujino S, Andoh A, Bamba S, Ogawa A, Hata K, Araki Y et al. Increased expression of interleukin 17 in inflammatory bowel disease. *Gut* 2003; 52:65–70.

92. Zheng SG, Wang J, Wang P, Gray JD, Horwitz DA. IL-2 is essential for TGF-beta to convert naive CD4$^+$CD25$^-$ cells to CD25$^+$FoxP3$^+$ regulatory T cells and for expansion of these cells. *J. Immunol* 2007; 178:2018-27.

93. Smits HH, Engering A, van der Kleij D et al. Selective probiotic bacteria induce IL-10-producing regulatory T cells *in vitro* by modulating dendritic cell function through dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin. *J Allergy Clin Immunol* 2005; 115:1260–7.

94. Taylor AL, Dunstan JA, Prescott SL. Probiotic supplementation for the first 6 months of life fails to reduce the risk of atopic dermatitis and increases the risk of allergen sensitization in highrisk children: a randomized controlled trial. *J Allergy Clin Immunol* 2007; 119:184–91.

95. Mazmanian SK, Round JL, Kasper DL. A microbial symbiosis factor prevents intestinal inflammatory disease. *Nature* 2008; 453:620–5.

*Korn T, Bettelli E, Oukka M, Kuchroo VK. IL-17 and Th17 Cells. Annu Rev Immunol. 2009;27:485-517.*

## List of figures

**Figures 1 a,b,c.** *In vitro* production of IL-2, IL-4, IL-6, IL-10, IL-12, IL-17, IFN-γ and TGF-β. 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 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* 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 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 of induced CD25+FoxP3+ cells [A], and the induction of ROR-γt 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

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

Values are means ± 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 | Cell count (CFU)/mL | | | | | | | | | | | |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| | LAVRI-A1 | | LGG | | Bl536 | | 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
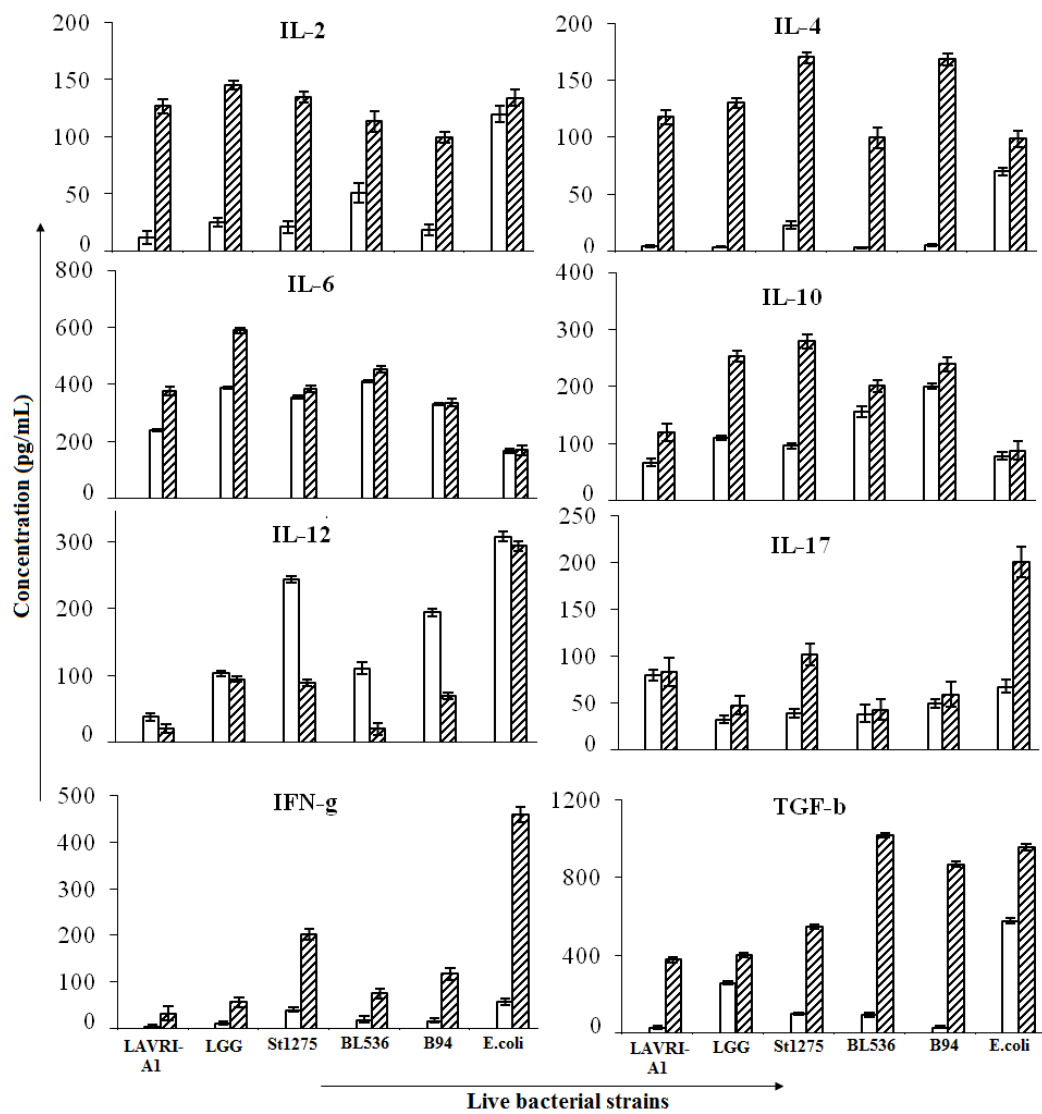
Figure: 1b GIT simulated bacteria induction of PBMC – pro- and anti-inflammatory cytokines
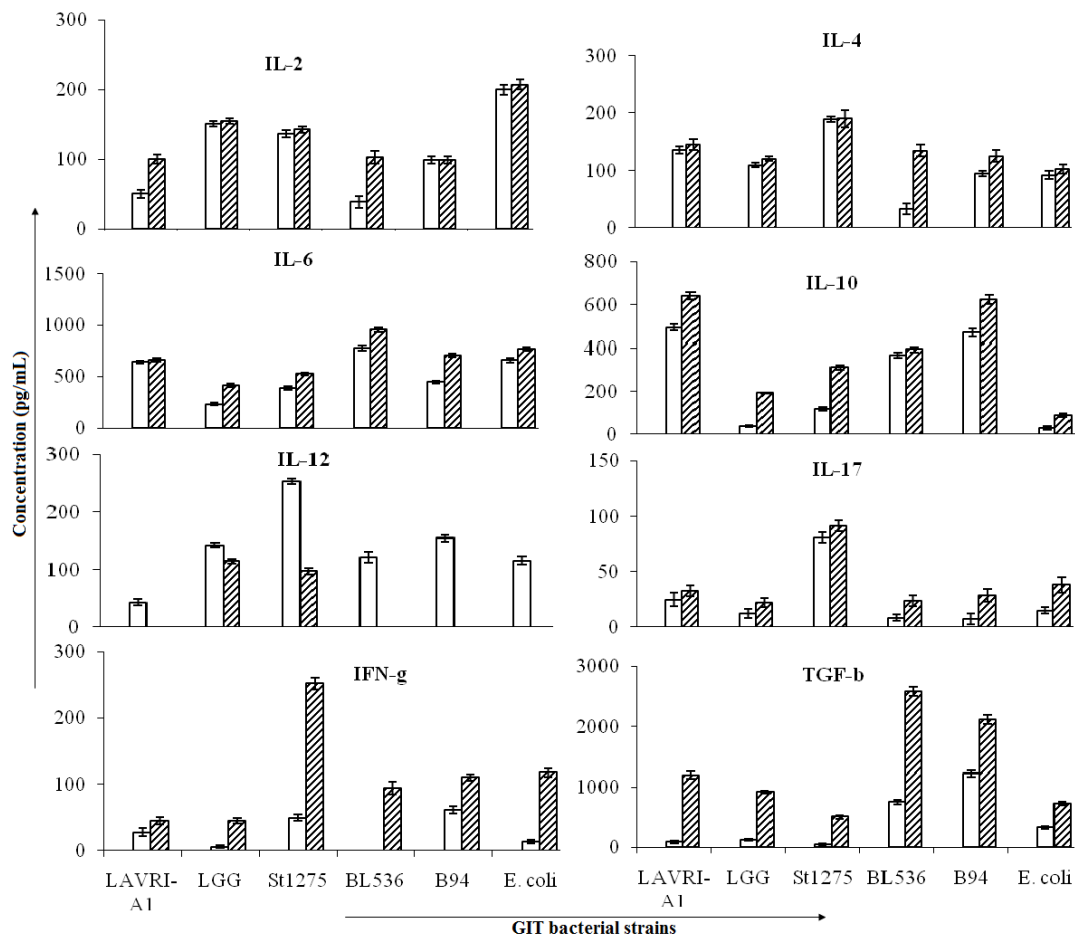
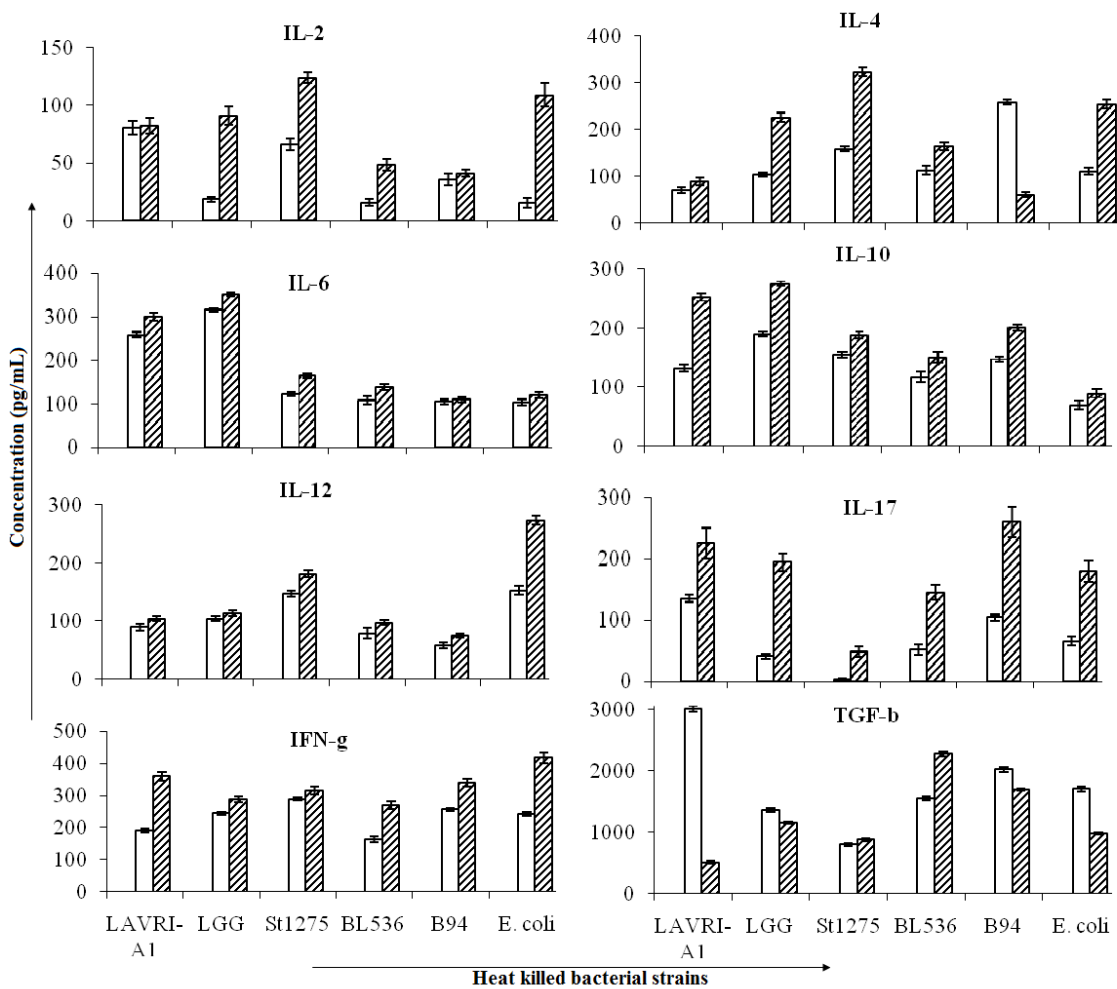Figure 1c Killed bacteria induction of PBMC – pro- and anti-inflammatory cytokines
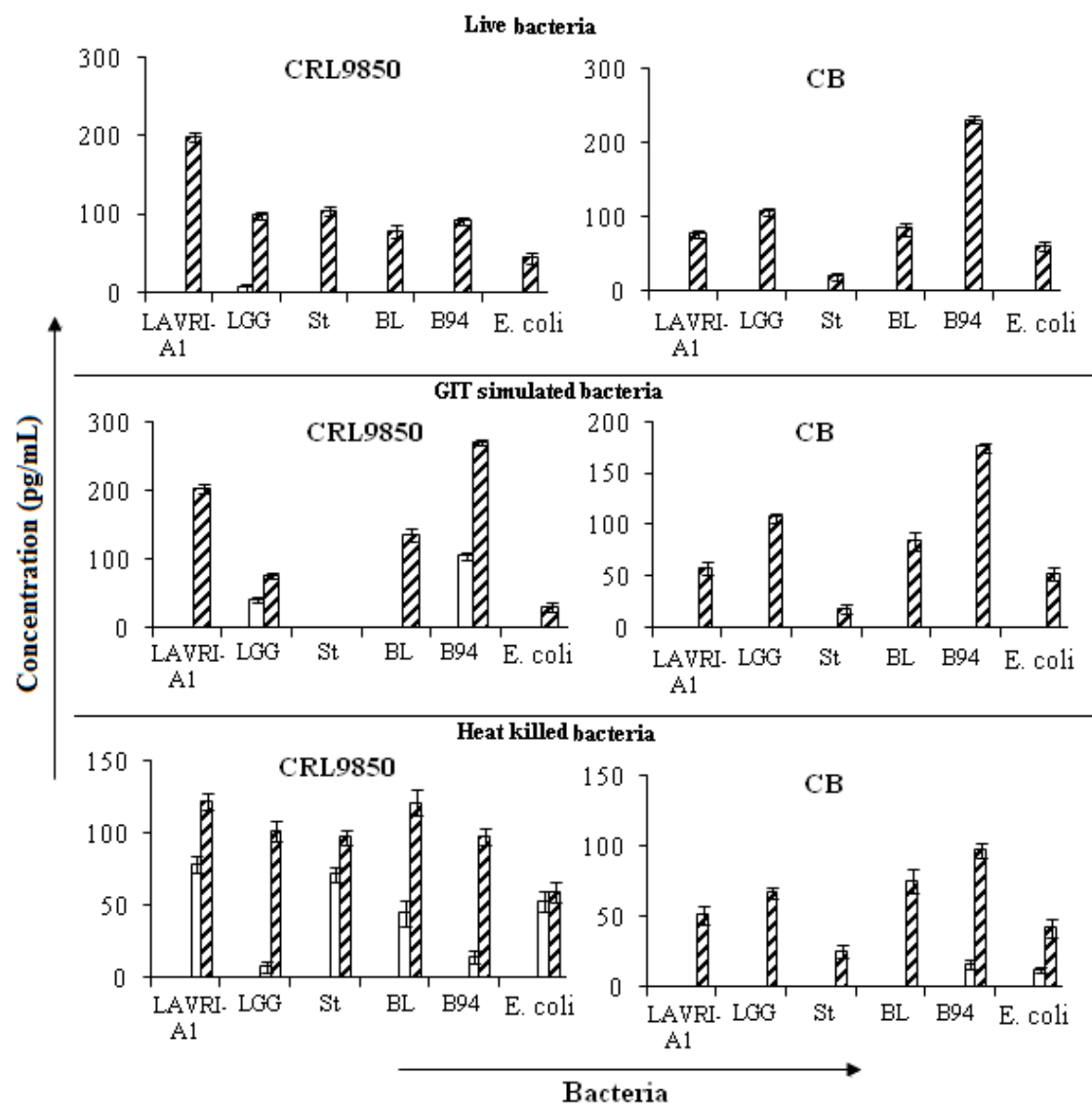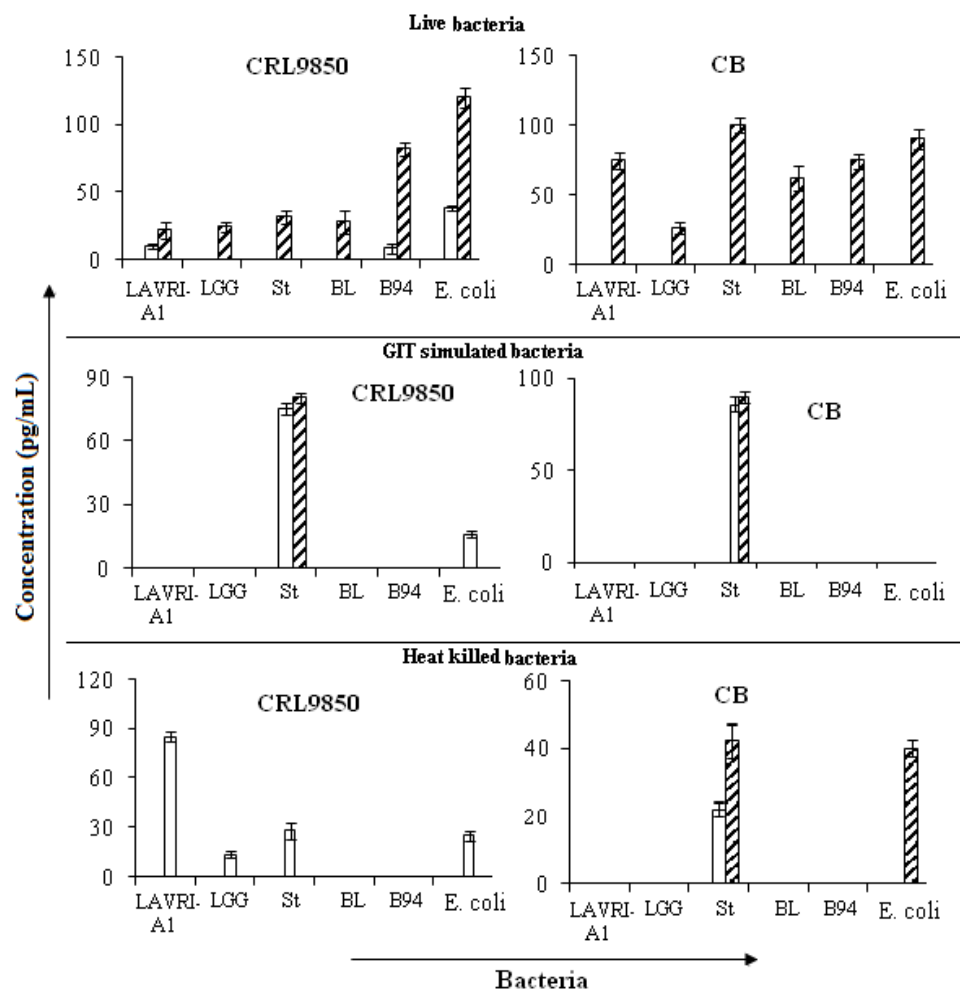
Figure 2a Production of IL-10
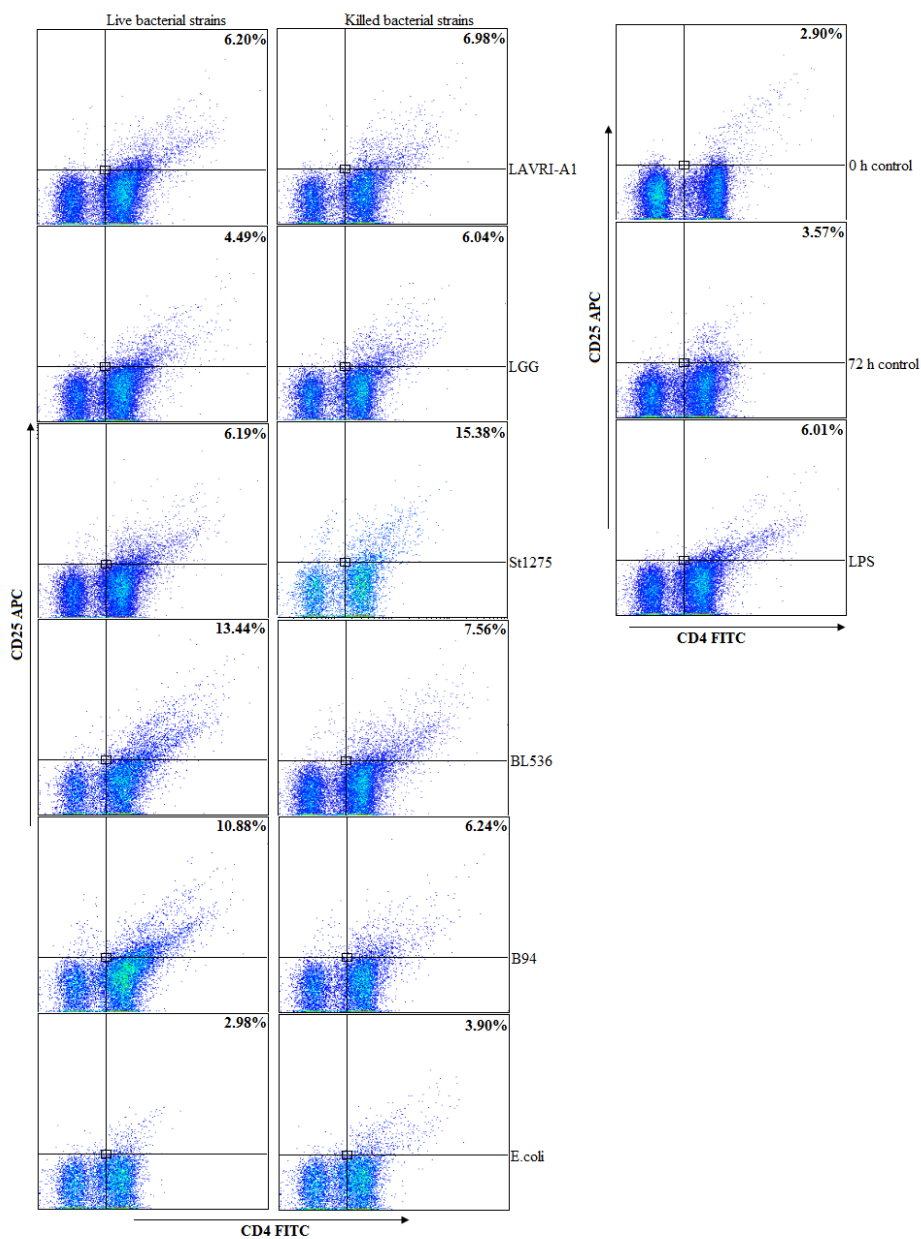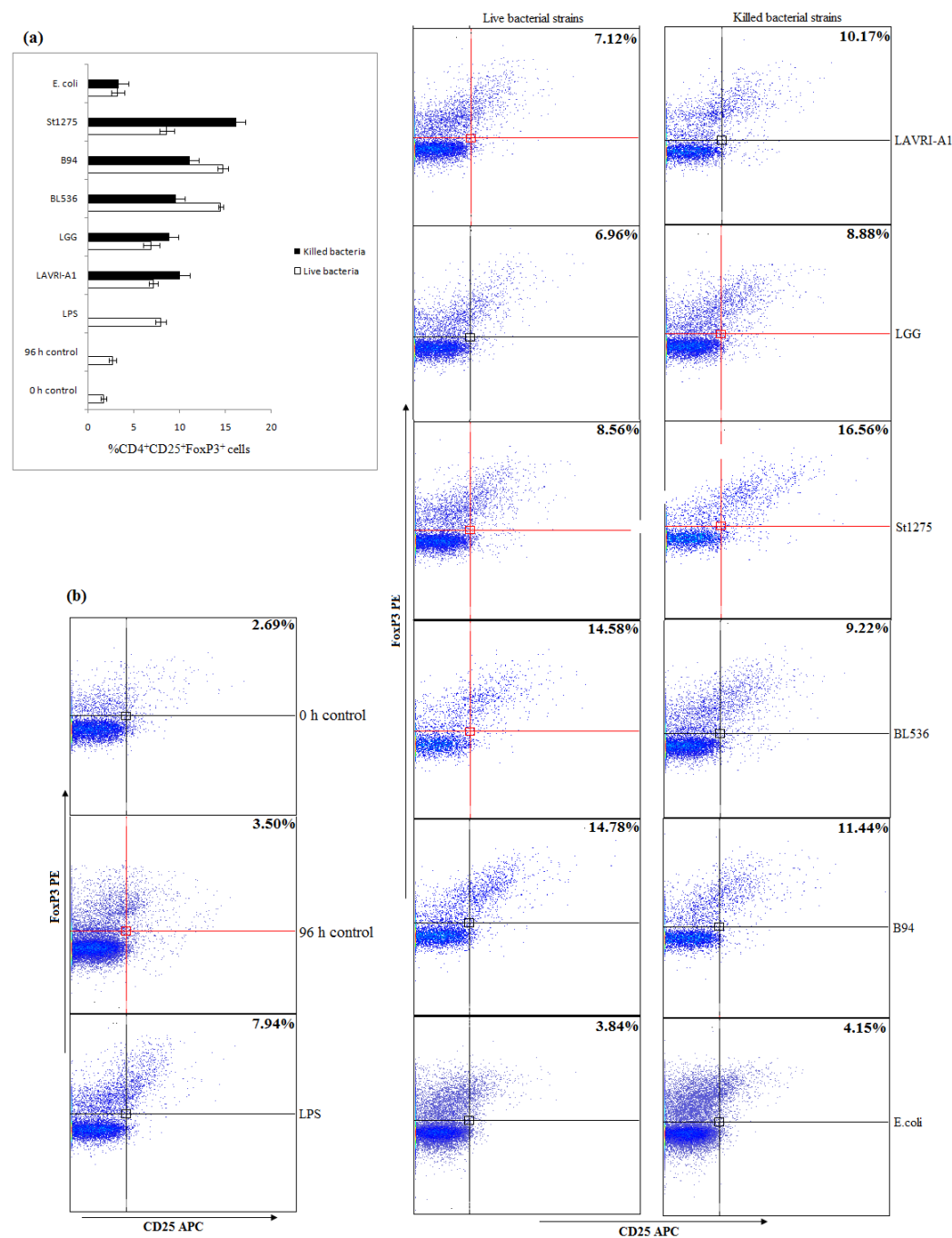
Figure 2b. Production of IL-17

Figure 3:

Figure 4

[A] a,b

[B] c,d

**(a)**



%CD3$^+$CD4$^+$CCR6$^+$RORgT$^+$ cells

**(b)**



RORgT PE

Live bacterial strains    Killed bacterial strains



CCR6 PerCp cy5.5

RORgT PE