

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

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Summary

The immunomodulatory effects of probiotics were assessed following exposure of normal peripheral blood mononuclear cells (PBMC), cord blood cells and the spleen-derived monocyte/macrophage cell line CRL-9850 to *Lactobacillus acidophilus* LAVRI-A1, *Lb. rhamnosus* GG, exopolysaccharides (EPS)-producing *Streptococcus thermophilus* St1275, *Bifidobacterium longum* BL536, *B. lactis* B94 and *Escherichia coli* TG1 strains. The production of a panel of pro- and anti-inflammatory cytokines by PBMC following bacterial stimulation was measured, using live, heat-killed or mock gastrointestinal tract (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; and (ii) cytokine levels increased relative to the expansion of bacterial cell numbers over time for cells exposed to live cultures. Bifidobacteria and *S. thermophilus* stimulated significant concentrations of transforming growth factor (TGF)- β , an interleukin necessary for the differentiation of regulatory T cells (T_{reg})/T helper type 17 (Th17) cells and, as such, the study further examined the induction of Th17 and T_{reg} cells after PBMC exposure to selected bacteria for 96 h. 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 potentially inducing differentiation of Th17 and T_{reg} cells.

Keywords: cytokines, T helper type 17, immunomodulation, probiotics, regulatory T cells

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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 be re-established with probiotic supplementation, consisting mainly 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 interleukin (IL)-2, tumour necrosis factor (TNF)- α and interferon (IFN)- γ , activate 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 [3]. Th17 cells are a subset of CD4⁺ T cells that produce a proinflammatory cytokine IL-17. Th17 cells have been shown recently to play a critical role in clearing pathogens during host defence reactions and in inducing tissue inflammation in autoimmune disease [4]. Regulatory T cells

(T_{reg}) are thought to be the master regulators of the immune response in both humans and rodents. Defects in the transcription factor forkhead box protein 3 (FoxP3), which defines the T_{reg} lineage, results in multiple autoimmune diseases and atopy [5,6], demonstrating the central role of FoxP3⁺ CD4 cells in immune homeostasis.

The probiotic, *Lactobacillus (Lb) rhamnosus* GG, has been shown to influence Th2-, Th1- and Th17-mediated disorders [7,8]. 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 by these strains [9]. The important discovery that transforming growth factor (TGF)- β and IL-6 could promote Th17 differentiation from naive T cells [10] prompted studies that confirmed that T_{reg} can also be generated *in vitro* by stimulation with TGF- β in the absence of IL-6 [11,12]. 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- β in controlling the newly described Th17 effector lineage and adaptive T_{reg} 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 [13]. Several studies have demonstrated that selected probiotics are able to induce the production of proinflammatory cytokines by macrophages and Th1 cytokines by peripheral blood monocytes [14,15]. 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 peripheral blood mononuclear cells (PBMCs) and the CRL-9850 cell line and the differentiation of Th17 or induced T_{reg} 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) were inoculated with 1% (v/v) LAVRI-A1 and LGG. Additionally, sterile 10 ml aliquots of MRS were

supplemented with 0.05% L-cystein.HCl and inoculated with 1% (v/v) B94 and BL536 and incubated at 37°C for 18 h. For the propagation of *E. coli* and St1275, 1% (v/v) 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 [16]. Following two successive transfers to fresh 10-ml broth preparations, bacteria were grown for 18 h log phase growth. Cultures were harvested at 1360 g for 30 min at 4°C. To heat kill, samples were incubated at 80°C for 30 min. GIT-simulated samples were treated as described below. Following these manipulations, preparations were centrifuged and the pellet resuspended in phosphate-buffered saline (PBS). Strains were washed three times in PBS and subsequently frozen at -80°C in aliquots of 10⁷ 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% anti-mycotic solution (Sigma).

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 [17,18] was used with some modifications, as described. To simulate bacterial digestion in the stomach, distilled-deionized 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 0.1 M 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 for 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 0.1 M NaHCO₃, pH 5.2 was added and incubated for an additional 2 h in the 37°C shaking water-bath. After a subsequent 10 min incubation on ice, the pH was adjusted to 7.2 and samples were centrifuged (1360 g for 15 min, 4°C) and the pellets washed in PBS, before resuspending in 30 ml PBS.

Enumeration of bacterial cells

For enumeration of bacterial cell numbers, 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*), M17 agar (St1275), de Man, Rogosa and Sharpe (MRS) agar (LAVRI-A1 and LGG) and MRS agar supplemented with 0.05% L-cysteine.HCl (bifidobacteria), and

incubated anaerobically for 72 h at 37°C [19]. 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 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

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's Hospital, Melbourne, Australia) by Ficoll-Paque gradient. PBMCs were isolated according to the methods described by Hesse *et al.* [13] and de Rooij *et al.* [20], with minor modifications. Briefly, buffy coats were diluted with an equal volume of PBS and layered on Ficoll-Paque Plus (GE Healthcare, Bio-Sciences, Uppsala, Sweden). Cells at the interphase were collected following centrifugation (680 g, 25 min, 18°C) (Sorvall® RT7 centrifuge; DuPont, Newtown, CT, USA). Blood lymphocytes were washed once in cold PBS, and following centrifugation (680 g, 18°C, 10 min) the pellet was resuspended in 2 ml red blood cell lysis buffer [0.15 M NH₄Cl, 0.01 M KHCO₃ and 10 µM ethylenediamine tetraacetic acid (EDTA) Na₂·2H₂O] and incubated for 2 min at 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 (10 mg/l; Sigma).

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

PBMCs/CRL-9850 cells were plated in six-well tissue culture plates (Corning, Sigma) at 5×10^6 cells/well and incubated at 5% CO₂, 37°C for 24 h prior to stimulation with bacteria, as described by Amrouche *et al.* [21]. Briefly, 10^6 freshly prepared viable (live or GIT) or equivalent ($\sim 10^6$ CFU/ml) heat-killed bacteria were added per 10^6 cells and co-cultured for 72 h at 5% CO₂, 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 (Becton Dickinson, San Jose, CA, USA), 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.

Cell staining and flow cytometry analysis

T_{reg}/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% CO₂ for 96 h, then cells were washed twice using FACS buffer (PBS + 2% FCS) and centrifuged at 500 g for 10 min. PBMC were resuspended at 10^6 cells/ml, and surface marker staining was performed using fluorescein isothiocyanate (FITC)-labelled anti-human CD4, allophycocyanin-labelled anti-human CD25/CD3 (Becton-Dickinson), peridinin chlorophyll protein (PerCP)-labelled anti-human CD3 (Biolegend, San Diego, CA, USA) and PerCP cyanine (Cy)5.5-labelled anti-human CCR6 (CD196). Intracellular staining was performed using phycoerythrin (PE)-labelled anti-human FoxP3/RORγt (BD Pharmingen and R&D Systems, Minneapolis, MN, USA, respectively), according to the manufacturer's instructions. Samples were read using a BD FACSCalibur, data acquired using CellQuest program (Becton Dickinson Biosciences), and analysis performed using Gatelologic version 3.07 software (Inivai, Victoria, Australia). Absolute numbers of T_{reg} 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 three main factors: strains (six levels) and treatments (three levels) as the main plot and time (five levels) as a subplot. The statistical evaluations of the data were performed using the general linear model (GLM) [22]. 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 Figs 1a–c and 2a,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 (Table 1a–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 [23]. Even though some cytokines were not detectable from individual donors, substantial and significant production of all investigated 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

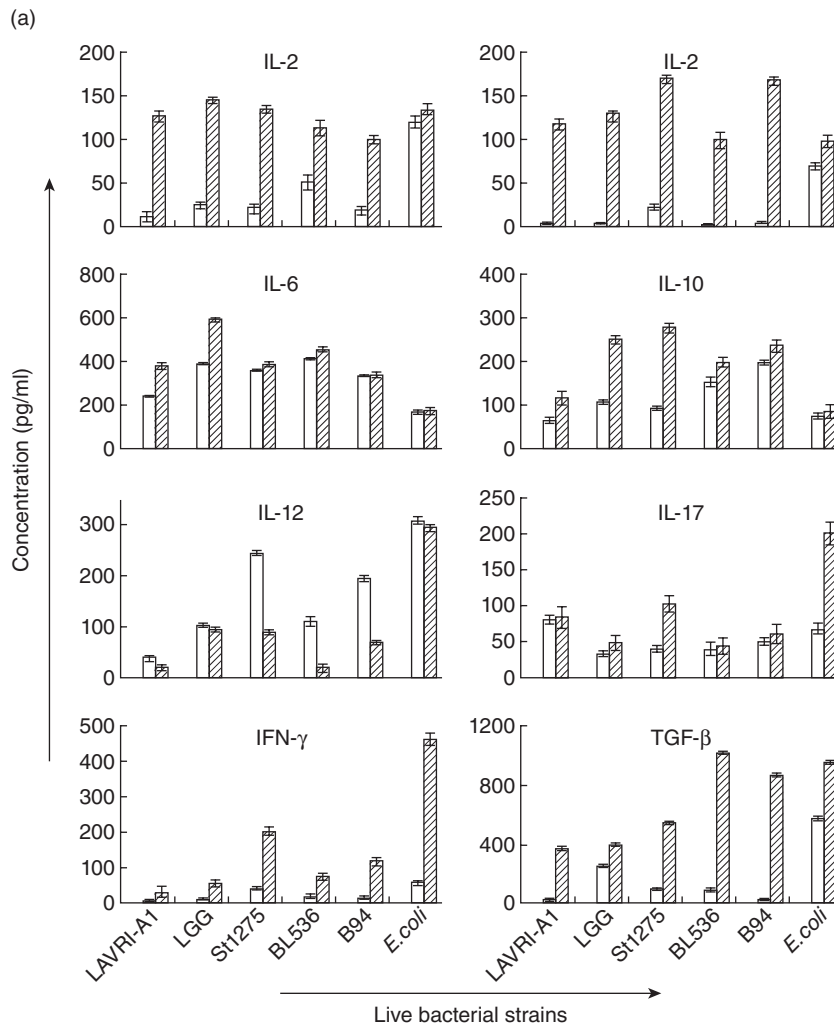


Fig. 1. (a–c) *In vitro* production of interleukin (IL)-2, IL-4, IL-6, IL-10, IL-12, IL-17, interferon (IFN)- γ and transforming growth factor (TGF)- β . Supernatants of \square 48 and \square 72 co-cultures of pooled buffy coat-derived peripheral blood mononuclear cells (PBMC) with live, gastrointestinal tract (GIT)-simulated and heat-killed *Lactobacillus acidophilus* LAVRI-A1, *Lb. rhamnosus* GG, exopolysaccharides (EPS)-producing *Streptococcus thermophilus* St1275, *Bifidobacterium longum* BL536, *B. lactis* B94 or *Escherichia coli* TG1 or PBMC in medium alone were collected. The concentration of cytokines was subsequently determined using enzyme-linked immunosorbent assay (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 (s.e.m.).

depending on the strain, as reported similarly by Wu *et al.* [24]. Generally, buffy coat-sourced PBMC produced 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 (6 h, data not shown) and maintained up to 72 h, while cord blood PBMC and CRL 9580 cells showed a later appearance of cytokines in culture (48–72 h, Fig. 2a,b), the delayed response due probably to a lack of established adaptive immune responses in cord blood [25]. While proinflammatory cytokines were produced significantly 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) [25,26]. 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 fetal immunoprotection [27,28].

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 significant differences were observed between the two sets of results, indicating that the bacteria are able to withstand the harsh physiological conditions (Table 2) [17,29]. Proinflammatory cytokine production was measured fol-

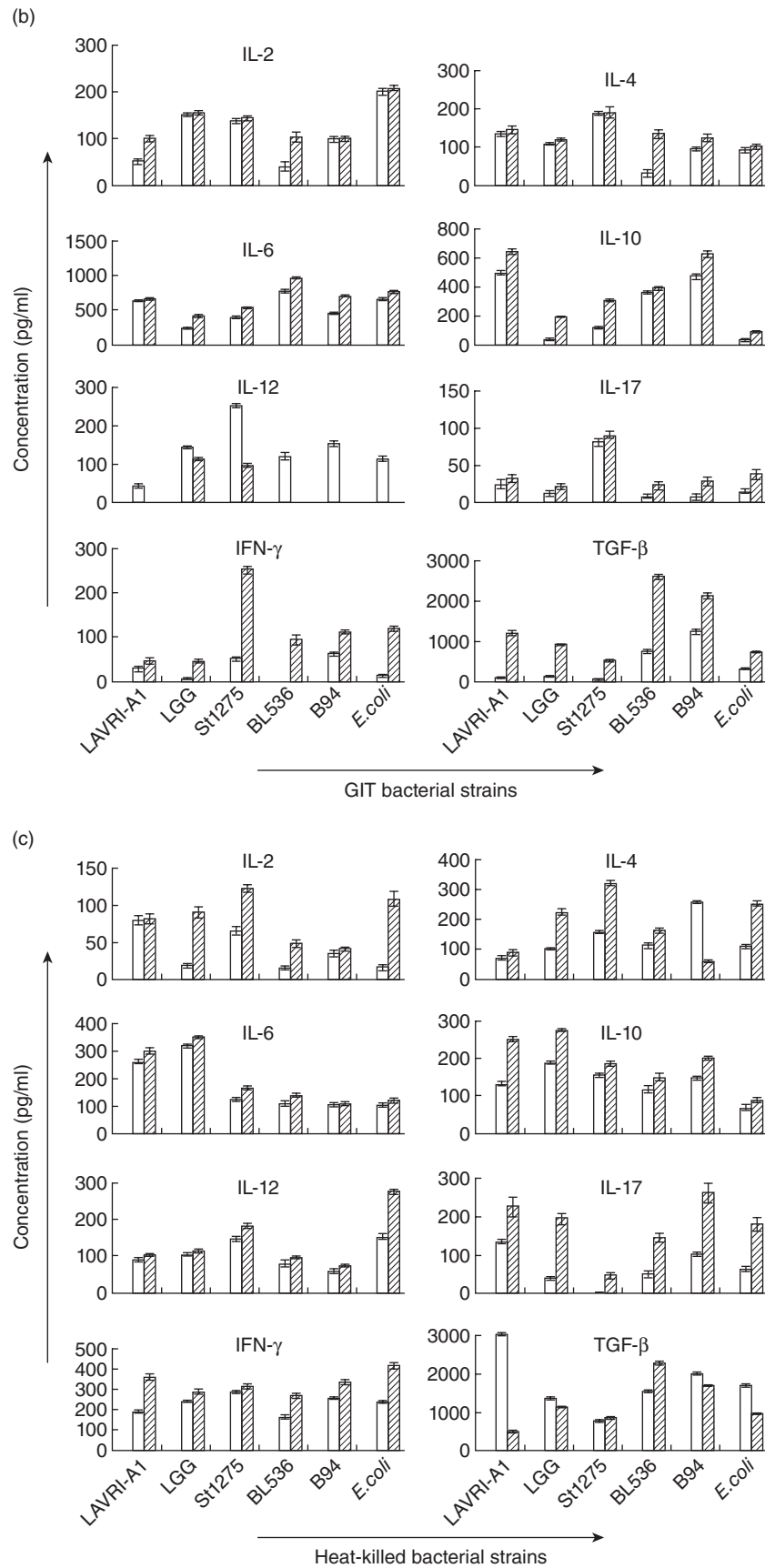


Fig. 1. *Continued*

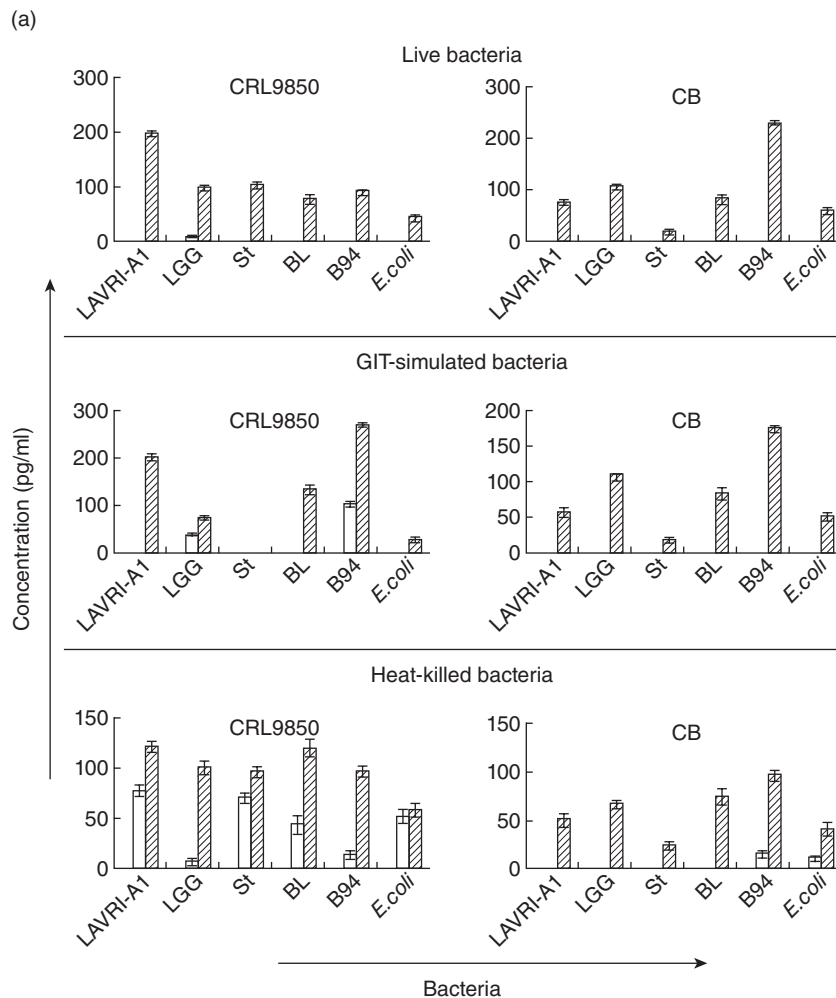


Fig. 2. (a,b) *In vitro* production of interleukin (IL)-10 and IL-17. Supernatants of \square 48 and \square 72 h co-cultures of CRL9850 or cord blood-derived peripheral blood mononuclear cells (PBMCs) with live, gastrointestinal tract (GIT)-simulated or killed *Lactobacillus acidophilus* LAVRI-A1, *Lb. rhamnosus* GG, exopolysaccharides (EPS)-producing *Streptococcus thermophilus* St1275, *Bifidobacterium longum* BL536, *B. lactis* B94 or *Escherichia coli* TG1 or PBMC in medium alone were collected. The concentration of cytokines was subsequently determined using enzyme-linked immunosorbent assay (ELISA) kits. Data are expressed as the mean cytokine response minus controls (pg/ml) of each treatment from triplicate wells, plus or minus standard error of the mean (s.e.m.).

lowing 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 (Fig. 1a,b). Of all the bacterial strains assessed, St1275 induced the highest production 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 concentrations 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 and 2b). *E. coli* induced IL-10 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

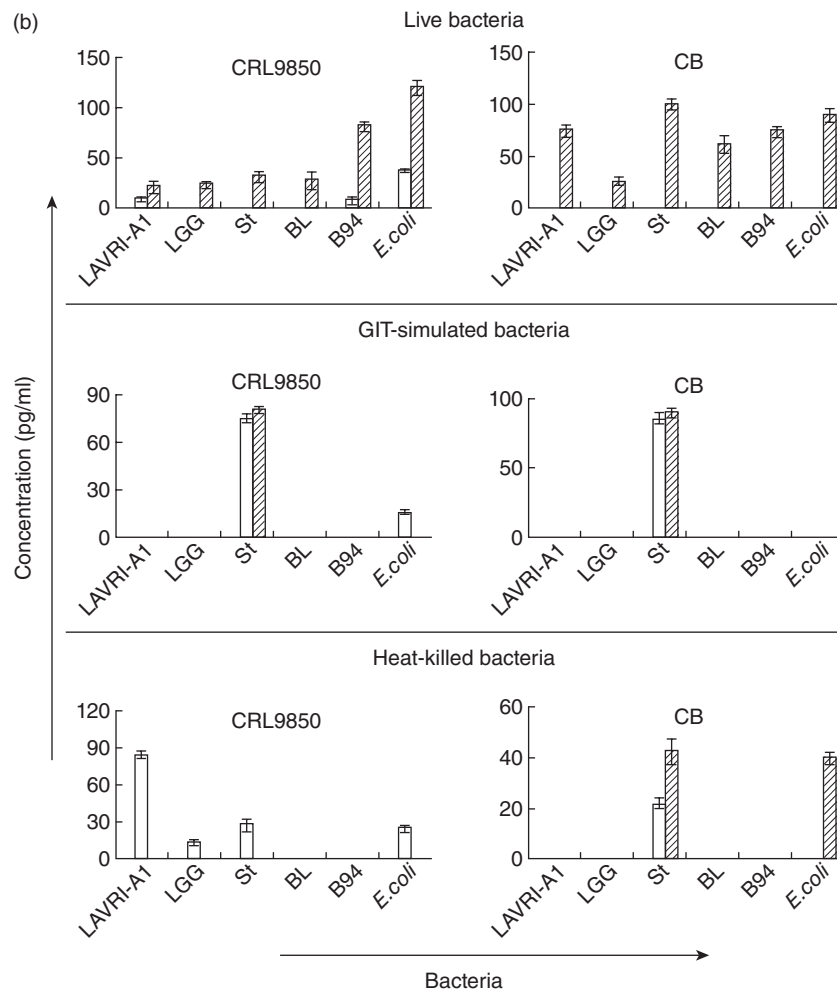
blood-derived PBMCs, only LAVRI-A1, LGG, BL536 and B94 induced significant ($P < 0.05$) levels of IL-10 production (Fig. 2a).

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 amounts ($P < 0.05$) when killed bacteria were incubated with the other cell types.

Induction of FoxP3 and ROR- γ t 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

Fig. 2. *Continued*

et al. [23]. To investigate whether treatment with lactobacilli or bifidobacteria lead to enhanced Th17 or T_{reg} cell differentiation we assessed Th17/T_{reg} populations in PBMC following 72–96 h of treatment with live or heat-killed bacteria. In all cases, following 72–96 h co-culture the number of T_{reg} (CD4⁺CD25⁺FoxP3⁺) cells as a percentage of total PBMC increased substantially compared to untreated control cells, albeit to different levels [Fig. 4a(i) and a(ii)]. 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-producing cells from PBMC was also investigated. As shown in Fig. 4b, the number of IL-17-expressing CD3⁺CD4⁺ cells was increased substantially compared to control. Because 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- γ t were subsequently used. The proportion of Th17 cells (CD3⁺CD4⁺CCR6⁺ROR- γ t⁺) induced by live and killed bacteria was increased 2.5-fold above control [Fig. 4b(i) and

b(ii)], with BL536 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

Probiotic bacteria are commonly marketed to aid digestion and optimize 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 [17,30]. Our results demonstrated the bacteria were resistant to the extreme conditions faced in the gut, in line with previous reports [17].

The current studies assessed the ability of common probiotics to induce cytokine production from PBMCs, cord blood cells and spleen-derived macrophages. The substantial

Table 1a. Cytokine levels of cultured peripheral blood mononuclear cells (PBMCs) in response to live lactic acid bacteria (LAB).

Donor	Strains	IL-2			IL-4			IL-6			IL-10			IL-12			IL-17			IFN- γ			TGF- β		
		48 h	72 h	48 h	48 h	72 h	48 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	48 h	72 h		
1	La	4.1 ± 1.7	n.d.	5.4 ± 1.5	10.7 ± 2.4	20.6 ± 5.2	14.4 ± 3.1	15.6 ± 4.4	n.d.	56.8 ± 15.7	32 ± 5.1	32.5 ± 41.3	41.8 ± 10.6	n.d.	22.64 ± 5.64	7 400 ± 1 264									
		22.4 ± 4.8	67.1 ± 20.2	1.4 ± 0.4	n.d.	182 ± 41	23.2 ± 2.9	34.4 ± 8.5	n.d.	n.d.	68 ± 12.3	116 ± 20	18.4 ± 1.4	34.5 ± 5.3	1246 ± 287	2 350 ± 525									
	Bl	13.5 ± 3.1	37.4 ± 9.2	47.1 ± 12.1	5.4 ± 1.7	21.3 ± 3.3	6.4 ± 1.3	24 ± 8.3	135 ± 32	29.3 ± 3.5	n.d.	18 ± 1.5	20.9 ± 5.2	60.4 ± 5.8	1478 ± 324	4 300 ± 423									
		18.2 ± 2.6	32.9 ± 8.1	21.8 ± 45.3	23 ± 6.1	50.3 ± 12.8	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	33.2 ± 63.8	23.14 ± 22.5	46.8 ± 14.3	87.2 ± 20.6	n.d.	149.7 ± 31.2	2200 ± 234	7.6 ± 1.6	86.9 ± 9.8	910 ± 154	9 750 ± 2 460									
		42.0 ± 12.5	33.8 ± 7.8	n.d.	n.d.	44.3 ± 13.8	n.d.	n.d.	n.d.	n.d.	12 ± 0.8	18 ± 1.4	n.d.	n.d.	850 ± 206	550 ± 57									
2	La	n.d.	n.d.	1.4 ± 0.6	10.4 ± 2.9	12.2 ± 3.3	13.1 ± 2.6	n.d.	61.3 ± 11.2	117.0 ± 28.6	8 ± 1.5	n.d.	15.1 ± 3.3	58.5 ± 6.1	205 ± 41	5 050 ± 1 263									
		12.3 ± 2.9	42.2 ± 10.2	1.4 ± 0.6	7.9 ± 2.3	147 ± 28	97.2 ± 20.4	n.d.	n.d.	15.8 ± 3.2	115.2 ± 25.4	n.d.	24.7 ± 8.1	37.6 ± 4.7	185 ± 14	215 ± 42									
	Bl	25.9 ± 5.6	n.d.	39 ± 11	n.d.	129 ± 24	76.9 ± 19.8	n.d.	32.4 ± 6.9	58.4 ± 8.2	384 ± 82	36 ± 2.8	24.6 ± 3.8	66.6 ± 5.2	4865 ± 1204	500 ± 62									
		8.8 ± 2.3	n.d.	11.4 ± 2.9	7.1 ± 1.9	90.3 ± 22.6	23.8 ± 6.9	18.7 ± 5.1	100.0 ± 15.4	38.4 ± 5.4	100.0 ± 15.4	42 ± 12.5	36.2 ± 6.2	49.3 ± 6.7	4555 ± 671	1 250 ± 429									
	St	9.4 ± 1.8	20.6 ± 6.3	15.4 ± 3.8	n.d.	128 ± 18	168.2 ± 37.1	73.2 ± 21.3	18.4 ± 4.8	n.d.	80 ± 11.4	74 ± 6.8	19.2 ± 1.7	50 ± 11.2	415 ± 41.3	18 350 ± 5 410									
		11.28 ± 3.5	33.8 ± 10.2	26 ± 4	n.d.	520 ± 108	67.6 ± 9.1	n.d.	n.d.	92.5 ± 17.5	41.5 ± 5.7	n.d.	131 ± 2.2	n.d.	300 ± 44	24 300 ± 4 630									
3	La	n.d.	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	10.8 ± 1.4	7.6 ± 12.1	11 ± 2.1	12 ± 3.6	60 ± 5.6									
		1.8 ± 0.4	n.d.	5.3 ± 1.8	10 ± 2.5	23.3 ± 5.7	8 ± 3.1	13.6 ± 2.8	73.5 ± 18.3	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	42.5 ± 6.5								
	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	54.1 ± 14.4	8.1 ± 1.7	40.6 ± 3.2	n.d.	n.d.	2.5 ± 3.8	16.0 ± 2.8	16.5 ± 2.6									
		25.9 ± 12.5	n.d.	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	41.0 ± 5.2	8.6 ± 1.2	20.5 ± 5.6	28.4 ± 9.2	70 ± 22	12.5 ± 3.3								
	St	2.4 ± 1.1	n.d.	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	12.5 ± 2.5								
		34.4 ± 11.4	42.2 ± 13.3	n.d.	n.d.	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	31.8 ± 4.4	40 ± 2.9	4 ± 1.3	n.d.	n.d.								

IL: interleukin; n.d.: not done; IFN: interferon; TGF: transforming growth factor.

Table 1b. Cytokine levels of cultured peripheral blood mononuclear cells (PBMCs) in response to gastrointestinal tract (GIT) lactic acid bacteria (LAB).

Donor	Strains	IL-2			IL-4			IL-6			IL-10			IL-12			IL-17			IFN- γ			TGF- β		
		48 h	72 h	48 h	48 h	72 h	48 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	48 h	72 h		
1	La	6.6 ± 1.2	30 ± 5.3	n.d.	n.d.	52.8 ± 13.2	124 ± 22	90 ± 12.6	78 ± 21.4	57.5 ± 13.6	52.5 ± 10.5	8 ± 2.4	152 ± 24	2.1 ± 0.5	12.8 ± 2.6	2180 ± 342	9400 ± 2546								
		7.5 ± 2.8	16.6 ± 4.2	n.d.	n.d.	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	n.d.	28.5 ± 5.9	n.d.	62.5 ± 9.7	41 ± 6.8	24.2 ± 4.4	57.0 ± 8.3	36 ± 2.9	n.d.	n.d.	n.d.	350 ± 41	3800 ± 465								
		n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	87.5 ± 12.6	43.2 ± 10.2	n.d.	n.d.	2.4 ± 1.1	4.7 ± 0.6	348 ± 33	410 ± 75								
	St	18.8 ± 5.2	19.7 ± 3.3	n.d.	n.d.	104.5 ± 22.5	520 ± 132	222 ± 4.5	800 ± 156	n.d.	n.d.	504 ± 46	90 ± 15.6	14.7 ± 3.5	41.8 ± 12.2	1100 ± 204	6550 ± 524								
		n.d.	n.d.	91.4 ± 21.5	n.d.	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	18.2 ± 4.8	87.2 ± 14.6	5450 ± 1425	1300 ± 156								
2	La	28.8 ± 12.3	38.8 ± 6.3	n.d.	n.d.	25.2 ± 6.43	211.8 ± 34.5	21.8 ± 5.6	67.0 ± 13.6	22.5 ± 6.5	8.4 ± 2.3	n.d.	n.d.	5.7 ± 2.6	15.4 ± 3.4	700 ± 133	1050 ± 245								
		12.2 ± 2.6	24.1 ± 12.5	n.d.	n.d.	90.1 ± 12.4	84.3 ± 10.6	27.5 ± 6.4	100 ± 22	13.2 ± 2.5	53.2 ± 15.4	n.d.	n.d.	14.2 ± 3.8	27.1 ± 6.2	350 ± 62	500 ± 85								
	Bl	28.8 ± 11.2	26.4 ± 5.8	n.d.	n.d.	76.8 ± 12.9	478 ± 56	8.5 ± 1.4	87.5 ± 32.5	2.25 ± 3.5	30 ± 5.9	n.d.	n.d.	7.8 ± 1.9	11.4 ± 2.4	128 ± 24	250 ± 36								
		50.8 ± 13.6	41.2 ± 6.9	n.d.	n.d.	61.1 ± 12.4	250.4 ± 35.6	158.2 ± 31.4	47.2 ± 7.8	72.7 ± 21.2	n.d.	n.d.	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	n.d.	90.5 ± 14.5	818 ± 146	13.8 ± 3.6	107 ± 17	1.8 ± 0.8	3.4 ± 0.8	27.0 ± 2.5	226 ± 53	22.8 ± 4.6	70 ± 10.6	4100 ± 654	7350 ± 1545								
		n.d.	n.d.	n.d.	n.d.	158.8 ± 41.6	957 ± 178	n.d.	n.d.	16.4 ± 3.3	4.5 ± 5.6	32 ± 8.4	n.d.	n.d.	21.44 ± 3.6	3150 ± 224	1300 ± 362								
3	La	25.5 ± 5.9	13.2 ± 3.6	n.d.	n.d.	11.4 ± 4.2	17.1 ± 13.6	5 ± 1.2	9.3 ± 2.8	n.d.	n.d.	48 ± 6.7	2.3 ± 0.7	1.8 ± 0.4	280 ± 25	1350 ± 254									
		12.9 ± 4.1	43.4 ± 12.5	n.d.	n.d.	7.1 ± 2.4	42.8 ± 12.5	3.2 ± 0.5	n.d.	n.d.	8 ± 1.4	4.4 ± 1.2	6.3 ± 1.4	147.5 ± 45.3	3350 ± 245										
	Bl	7.7 ± 2.3	3.6 ± 0.9	n.d.	n.d.	21.4 ± 3.6	19.8 ± 3.4	1.8 ± 0.4	2.4 ± 0.6	100.0 ± 21.2	28.4 ± 4.4	n.d.	n.d.	5.1 ± 1.2	2.9 ± 0.6	72.5 ± 45	1584 ± 128								
		1.66 ± 2.9	42.8 ± 11.3	14.2 ± 3.5	39.4 ± 6.7	50 ± 8.5	78.5 ± 19.4	n.d.	n.d.	110.7 ± 15.8	38.2 ± 5.1	n.d.	n.d.	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	74.5 ± 13.5	170 ± 32.4	187 ± 22	121.7 ± 32.4	43.2 ± 5.8	128 ± 5.63	106 ± 25.6	4.4 ± 1.5	12.3 ± 2.4	350 ± 34	7350 ± 2547								
		90 ± 32.5	47.5 ± 10.5	12.9 ± 2.5	70 ± 21	11.4 ± 4.1	37.1 ± 6.9	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	4.23 ± 0.68	284 ± 62									

Table 1c. Cytokine levels of cultured peripheral blood mononuclear cells (PBMCs) in response to heat-killed lactic acid bacteria (LAB).

Donor	Strains	IL-2		IL-4		IL-6		IL-10		IL-12		IL-17		IFN- γ		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	n.d.	n.d.	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	12.1 ± 2.3	12.8 ± 32.5	74 ± 14	40 ± 11.8	12.8 ± 3.6	3100 ± 1510	4100 ± 1885
	Bl	n.d.	n.d.	35.1 ± 5.2	182 ± 33	135 ± 32.5	197 ± 35.6	12.8 ± 4.4	12.5 ± 3.5	30 ± 4.8	30 ± 4.8	12.9 ± 2.6	52 ± 12	110 ± 24.7	63.2 ± 16.4	4600 ± 1652	1300 ± 365
	B94	n.d.	n.d.	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	11.2 ± 2.4	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
2	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	30.8 ± 4.2	33.7 ± 8.4	51.9 ± 6.3	n.d.	n.d.	55.7 ± 12.2	14.2 ± 3.9	1600 ± 285	5400 ± 1425
	La	210 ± 35	82.3 ± 21.2	3.4 ± 0.8	1.9 ± 0.6	20.4 ± 36.8	192 ± 32.2	11.4 ± 4.5	40 ± 8	4.3 ± 1.2	9.6 ± 1.3	18.2 ± 2.8	66 ± 6.6	11.8 ± 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	n.d.	n.d.	95 ± 22.1	47.3 ± 6.4	10 ± 4	106 ± 22	14.3 ± 2.9	97.1 ± 24.6	450 ± 113	245 ± 82
	Bl	n.d.	n.d.	12.8 ± 4.4	208 ± 35	n.d.	n.d.	431 ± 108	55.4 ± 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	n.d.	n.d.	3.7 ± 0.5	n.d.	127 ± 18.6	235 ± 35	35.7 ± 6.8	23.4 ± 6.3	4.3 ± 0.8	n.d.	30 ± 6.5	14.6 ± 3.8	8.5 ± 1.7	12.4 ± 2.4	2700 ± 875	1300 ± 278
3	St	5.8 ± 1.7	14.1 ± 2.8	n.d.	n.d.	54.2 ± 13.6	118 ± 12	73.4 ± 12.4	12.1 ± 3.12	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	n.d.	27.1 ± 5.2	52.8 ± 8.5	28.5 ± 8.5	42.8 ± 8.4	25.8 ± 5.2	68.8 ± 19.8	n.d.	n.d.	216 ± 46	289 ± 42	48.5 ± 12.3	192 ± 46	8500 ± 2250	4300 ± 1250
	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	11.5 ± 21.5	40 ± 11.2	518 ± 7.8	12.1 ± 18	56.7 ± 13.3	5850 ± 1815	3050 ± 815
	LGG	18.8 ± 3.5	8.3 ± 3.4	n.d.	n.d.	16.4 ± 3.2	7.4 ± 2.6	96.6 ± 14.6	171 ± 52	67.5 ± 15.5	52.8 ± 8.8	60 ± 15.4	1140 ± 25.4	287 ± 52	132 ± 28	1250 ± 235	2350 ± 520
	Bl	45.5 ± 5.9	77.7 ± 15.4	55.7 ± 16.3	23.1 ± 4.6	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
4	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	n.d.	n.d.	12 ± 4	20 ± 4.2	288 ± 62	34.2 ± 6.8	1300 ± 220	2450 ± 425
	Ec	15.5 ± 2.9	23.3 ± 6.5	n.d.	n.d.	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* (La); *Ec*; *Escherichia coli*; TG1; IL; interleukin; IFN; interferon; TGF; transforming growth factor. *Streptococcus* (S); *thermophilus* S1275; *Escherichia* (E); coli; TG1; IL; interleukin; IFN; interferon; TGF; transforming growth factor.

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 [31,32]; if these effects are largely IL-12-driven, St1275, B94 and *E. coli* in our study may probably be as effective in their immunomodulatory effects. Miettinen *et al.* [15] reported that LGG induced the production of proinflammatory cytokines such as IL-6, IL-12 and IFN- γ but limited IL-10 from human PBMC. Conversely, in our study LAVRI-A1, LGG and bifidobacteria induced significantly higher concentrations of IL-10 from PBMCs compared to the proinflammatory 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 of proinflammatory cytokines, including IL-2, which is a critical cytokine for clonal expansion of recently antigen-activated T cells and in T_{reg} homeostasis [33]. 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 [34]. 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 [35]. IL-17 has been found recently to be elevated in the intestinal tissue and serum of patients with inflammatory bowel disease (IBD) and other autoimmune disorders [36]. 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 proinflammatory cytokines from antigen-presenting cells, including macrophages, as well by inducing expression of other co-stimulatory surface molecules and soluble cytokines [37]. 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 [14,38,39]. In addition to its activity as a Th2 lymphocyte cytokine, IL-10 is also a potent deactivator of monocyte/macrophage proinflammatory cytokine synthesis [40]. TGF- β 1 down-regulates monocyte and macrophage activity in a manner similar to IL-10, albeit less potently [41]. 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- β 1 knock-out mouse attests to the physiological role of TGF- β as an endogenous anti-inflammatory cytokine [42].

Even though in this study Gram-negative *E. coli* stimulated substantial amount of proinflammatory cytokines, the induction of pro- and anti-inflammatory cytokines with live

Table 2. Enumeration of bacteria after 18 h incubation.

Treatment	LAVRI-A1		LGG		BL536		B94		St1275		<i>E. coli</i>	
	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
s.e.m.	0.24											

Results presented as a mean ($n = 3$) \pm pooled standard error of the mean (s.e.m.) (0.243). *Lactobacillus* (*Lb.*) *acidophilus* LAVRI-A1; *Lb. rhamnosus* GG (LGG); *Bifidobacterium* (*B.*) *lactis* B94; *B. longum* BL536; exopolysaccharides (EPS)-producing *Streptococcus* (*S.*) *thermophilus* St1275; *Escherichia* (*E.*) *coli*; GIT: gastrointestinal tract; CFU: colony-forming units.

Gram-positive bacteria (including GIT simulated bacteria), on average, was significantly higher. Hessle *et al.* [13] reported that Gram-positive bacteria appeared to stimulate IL-12 production and Gram-negative bacteria stimulate IL-10 production preferentially. However, concordant with observations reported in Berg *et al.* [43] and in our study, Gram-negative *E. coli* induced the secretion of significant concentrations of proinflammatory 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 [43]. 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 [44], with the balance skewed towards the anti-inflammatory response in our

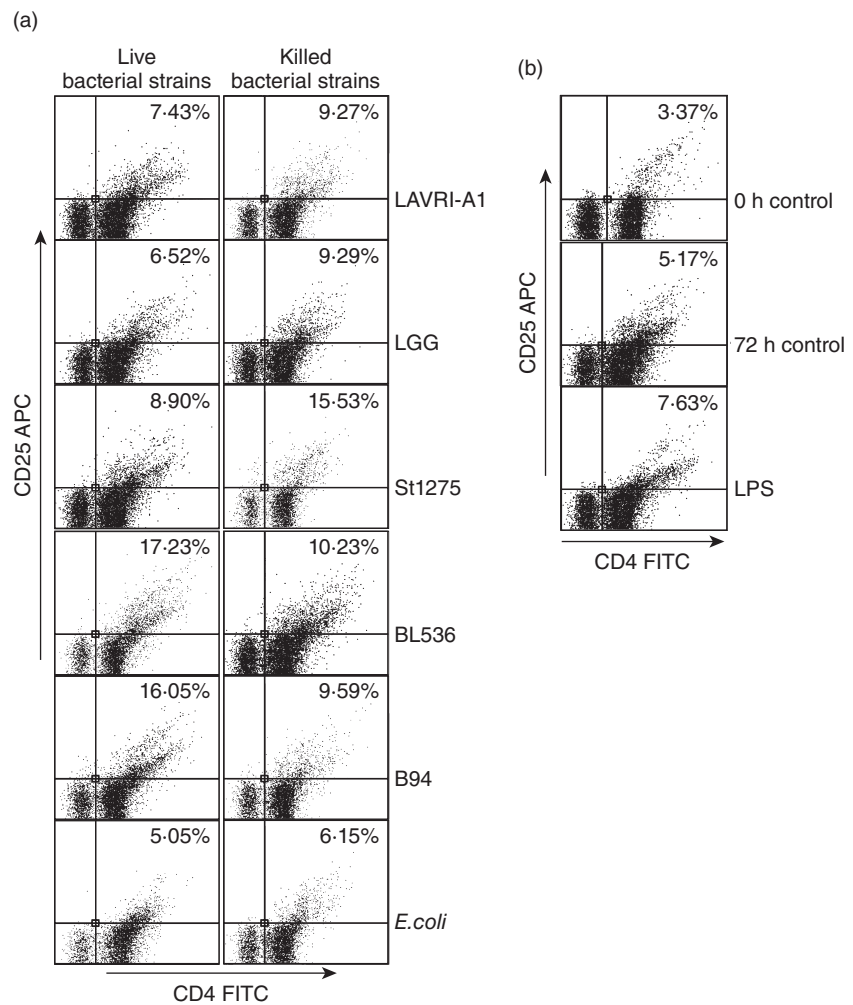


Fig. 3. Expression of activation marker CD25 by lymphocytes in response to selected bacteria. peripheral blood mononuclear cells (PBMCs) were cultured with *Lactobacillus acidophilus* LAVRI-A1, *Lb. rhamnosus* GG, exopolysaccharides (EPS)-producing *Streptococcus thermophilus* St1275, *Bifidobacterium longum* BL536, *B. lactis* B94 or *Escherichia 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.

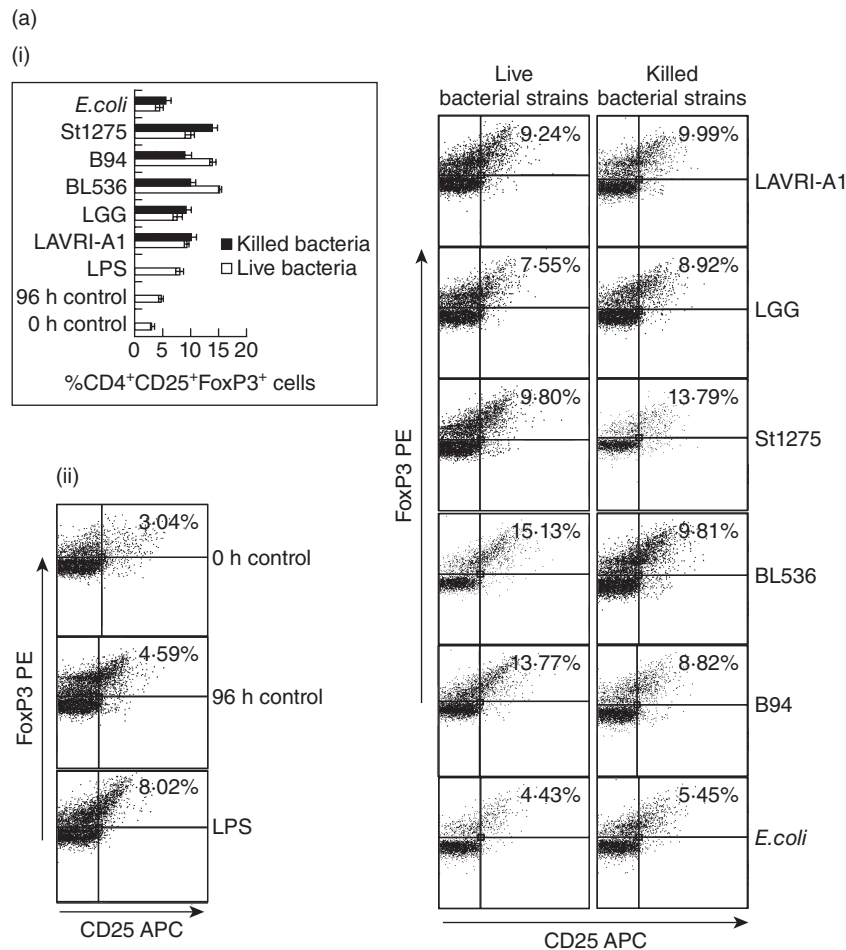


Fig. 4. Peripheral blood mononuclear cells (PBMCs) were co-cultured with live or heat killed *Lactobacillus acidophilus* LAVRI-A1, *Lb. rhamnosus* GG, exopolysaccharides (EPS)-producing *Streptococcus thermophilus* St1275, *Bifidobacterium longum* BL536, *B. lactis* B94 or *Escherichia coli* TG1 in a ratio that does not induce apoptosis, lipopolysaccharide (LPS) or cells alone as control. The percentage of induced CD25⁺forkhead box protein 3 (FoxP3⁺) cells (a), and the induction of ROR- γ t expressing T helper type 17 (Th17) cells (b), were assessed intracellularly by fluorescence activated cell sorter (FACS) Calibur 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 (s.e.m.) of three independent experiments.

study. A demonstration of the utility of this response is the finding that LGG reduced inflammation in Crohn's disease [45]. The human gut microbiota has been estimated recently to consist of at least 400 different species [46], 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 [23]. In addition, the effects of lactobacilli supplementation on experimental autoimmune encephalomyelitis have been shown to be highly strain-dependent [47]. It is therefore conceivable that the contradicting results found in the human trials can be explained partly 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 intracellular bacterial components as well as metabolites probably contribute to cytokine production [48]. 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 [21,49]. Cross *et al.* [50] and Macpherson and Harris [51] 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 immunomodulatory 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 influenced its ability to stimulate sustained and substantial levels of cytokines in the co-cultures. Exopolysaccharides from LAB have been claimed to partici-

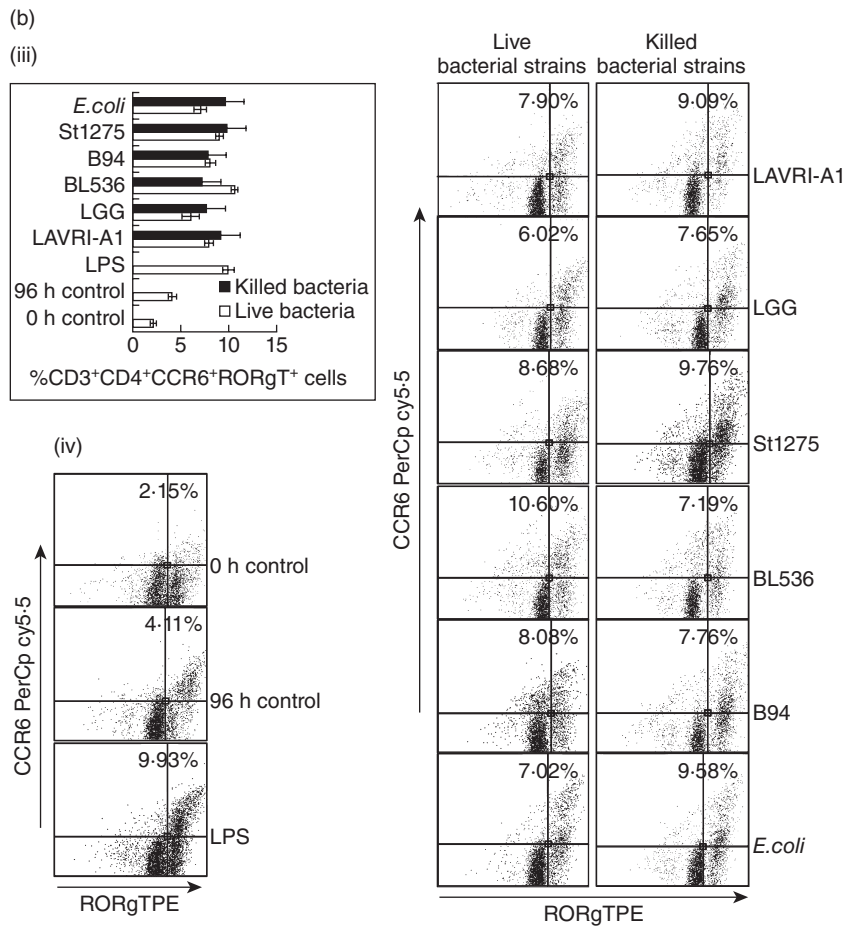


Fig. 4. Continued

pate in various regulatory processes such as immunomodulatory, cholesterol-lowering and anti-ulcer activities.

This study also investigated the differentiation of T_{reg} and Th17 cells from PBMCs stimulated with the bacteria. TGF-β has been shown to be involved in both T_{reg} and Th17 development. Animal models have demonstrated that at high levels of TGF-β, FoxP3 expression is up-regulated and T_{reg} differentiation is induced, whereas at low levels of TGF-β, IL-6 and IL-21 synergize to promote the differentiation of Th17 cells [52]. In the current studies, we observed elevated levels of TGF-β in the PBMC supernatant following incubation with the probiotics, suggesting a prime environment for T_{reg} differentiation. Indeed, substantially increased numbers of T_{regs} were identified in these cultures. Similarly, the identification of the transcription factor ROR-γt by intracellular and CCR6 extracellular staining confirmed the presence of Th17 cells. Th17 cells induce a range of pro-inflammatory mediators that bridge the innate and adaptive immune response enabling the clearance of invading pathogens [53]. The balance between T_{reg} and Th17 cells may be essential for maintaining immune homeostasis. Hence, therapeutic approaches that aim to re-establish homeostasis by increasing the number of T_{reg}, while also controlling effec-

tor 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 [20]. The mechanism of FoxP3⁺ T_{reg} 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 [54]. This was also apparent in our study, as 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 [20], when strains of lactobacilli differed significantly in their capacity to induce FoxP3⁺ regulatory cells *in vitro*, independent of the IL-10 production. The overall extent of induction of FoxP3⁺ (T_{reg}) 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 [55]. Previously, *Lb. acidophilus* strain LAVRI-A1 had no clinical effect on eczema [56]; however, this strain may be effective for other inflammatory disorders, as the current study shows a

moderate induction of FoxP3/ROR- γ t *in vitro*. Future studies will focus on the 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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Disclosure

The authors declare no conflicts of interest.

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