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Original article

Gut derived lactic acid bacteria induce strain specific CD4$^+$ T cell responses in human PBMC

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SUMMARY

Background & aims: Probiotic bacteria are used as food supplement in many different disease settings. The immune modulating capacity of different strains is not always properly tested which might result in a suboptimal choice of strains for clinical use.
Methods: The CD4 T cell responses to 19 different gut derived lactic acid bacteria were tested with different methods to show their diversity in immune modulation and to make a well-founded choice on which strains to use in future clinical trials.
After co-culture of PBMC with bacteria, the induction of CD4$^+$ T cell subsets (regulatory T cells, T helper type (TH)1, TH2 and TH17) was analysed by rtPCR of transcription factor mRNA, intracellular FACS staining of transcription factors and cytokine production.
Results: Bacterial strains all have diverse, unique immune modulatory properties. Strains can induce Treg, TH1, TH2 and TH17 cells which can be shown at different levels of T cell activation, and is consistent for most strains tested. For TH1, TH17 and Treg, a positive correlation between the different methods was found. For TH2 cells the correlation was less consistent.
Conclusions: Probiotic bacteria have very different immune modulating capacities. Analysis of transcription factor mRNA is a suitable method for *in vitro* characterization of strains prior to clinical application.

1. Introduction

Human gut derived bacterial strains are frequently used as probiotic supplements for a variety of medical affections, including diarrhoea, constipation, and infections.[1] One of the most challenging objectives of probiotic supplementation however, is to influence the immune system.[2] Dendritic cells residing in the lamina propria of the gut are influenced by food antigens and the gut microbiota by direct contact of dendrites sampling the gut lumen and by cytokine production of the epithelial cell layer. These dendritic cells will subsequently move to draining lymph nodes and activate T cells there.[3] The first experiments with supplementation of lactic acid bacteria were based on the hygiene hypothesis and had the objective to prevent or cure atopic diseases. In the original dogma of the TH1-TH2 balance, it was thought that probiotic bacteria will mainly induce T helper (TH) type 1 cells, thereby skewing the balance away from the TH2 cells which have an important role in allergic inflammation.[4,5]

However, in recent years, it became evident that T helper cell functions are considerably more complex and heterogeneous than originally thought. The original characterization of the TH1 and TH2 pathways has now been expanded to include additional TH cell subsets each with their own cytokine repertoire and transcription factors. These include TH9,[6] TH17[7] and FOXP3$^+$ regulatory T (Treg) cells.[8–11] These subsets also have a role in the initiation and

Abbreviations: cfu, colony-forming units; FACS, fluorescence activated cell sorting; FOXP3, forkhead box P3; GATA3, GATA binding protein 3; IL, interleukin; MACS, magnetic activated cell sorting; PBMC, peripheral blood mononuclear cells; RORγT, retinoic acid receptor-related orphan receptor γ; T-bet, T box expressed in T cells; Treg, regulatory T cell.

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augmentation of autoimmunity and allergy and have added more layers of complexity to the pathogenesis of such diseases. This enforces revisiting and reinterpreting existing ideas on the concept of probiotic supplementation as a therapeutic option for auto-immune and allergic diseases. Combating a TH2/TH17 mediated disease such as allergic asthma through immune regulatory actions of gut derived bacteria should not only involve the induction of TH1 cells but also the induction of FOXP3$^+$ Treg. In contrast, probiotics targeting rheumatoid arthritis or multiple sclerosis should inhibit TH1 and TH17 responses and promote the induction of TH2 and Treg cells.

Recent papers suggest that gut derived bacterial strains all have a different influence on the immune system.[12,13] To be able to select strains suitable for clinical supplementation, the immune influencing and other characteristics of each strain should be documented accurately.[14] The choice of strains should subsequently fit the experimental setup. We recently showed that the capacity to induce FOXP3$^+$ regulatory T cells is highly variable between strains. Some strains were able to induce significant numbers of FOXP3$^+$ T cells with clear regulatory function whereas other strains were not.[15] It is likely that the capacity of gut derived bacterial strains to either down regulate or induce the differentiation of other TH subtypes is just as variable.

To select potential strains for future clinical trials we investigated the immune influencing capacity of 19 bacterial strains of commonly used species using a high throughput method. The strains were co-cultured with peripheral blood mononuclear cells (PBMC) of healthy adult donors and the induction of the different CD4$^+$ T cell subtypes was evaluated on mRNA and protein level of signature transcription factors and cytokines. We show that the capacity of each strain to influence the immune system is highly different and that some strains have immune modulatory capacities that are favourable or not.

2. Materials and methods

2.1. Cells and bacteria

PBMC were isolated from healthy adult volunteers using Ficoll-Isopaque density gradient centrifugation (Ficoll-Paque, Pharmacia, Upsalla, Sweden). For some experiments, PBMC were depleted of CD25$^+$ cells using anti-CD25 magnetic beads (BD Biosciences, San José, CA, USA). PBMC or CD25$^-$ PBMC were subsequently co-cultured with bacteria in a 1:10 ratio (PBMC:CFU) in RPMI 1640 (Gibco, Breda, The Netherlands) supplemented with 1% L-glutamine (Gibco), 0.5% Penicillin-Streptomycin (Gibco) to prevent bacterial overgrowth and 10% human AB serum. The 1:10 ratio (based on viable bacteria) was shown before to induce T cell responses without apoptosis.[15] Bacterial viability was tested by FACS analysis using the BacLight-kit (Invitrogen, Breda, The Netherlands) Since the presence of CD4$^+$CD25$^+$ Treg within the total PBMC population inhibits proliferation of the T cells and protein synthesis, we increased the sensitivity of some tests by using CD25$^+$ cells depleted PBMC.

Bacteria (*Streptococcus thermophilus* W67, *Bacillus coagulans* W64, *Bifidobacterium bifidum* W28, *Bifidobacterium breve* W25, *B. breve* W29, *B. breve* W6, *Bifidobacterium lactis* W51, *B. lactis* W52, *Bifidobacterium longum* W108, *Lactobacillus acidophilus* W22, *L. acidophilus* W55, *L. acidophilus* W74C, *Lactobacillus casei* W20, *L. casei* W79, *Lactobacillus plantarum* W21, *L. plantarum* W62, *Lactobacillus rhamnosus* W71, *Lactobacillus salivarius* W57, *Lactococcus lactis* W19) were obtained from Winclove BioIndustries BV, Amsterdam, The Netherlands. Bacteria were frozen at −80 °C in RPMI 1640 (Gibco) supplemented with 1% glutamine (Gibco). For each experiment, a vial was thawed, diluted to the correct concentration and co-cultured with PBMC. Viability and bacterial cell number of the strains used was assessed by FACS using the BacLight-kit from Molecular Probes (Eugene, OR, USA).

For rtPCR, CD4$^+$ T cells were isolated from PBMC after 6 days of culture using a CD4 T cell enrichment kit (BD Bioscience). CD4 T cell purity was >95%. Cells were subsequently lysed in Tripure (Roche Diagnostics, Basel, Switzerland) and frozen at −80 °C.

2.2. rtPCR

mRNA was isolated from lysed CD4$^+$ T cells and cDNA prepared using the iScript cDNA synthese kit from BioRad (Hercules, CA, USA). cDNA was frozen at −20 °C till use for rtPCR. Primers for β2M (Fw: CCAGCAGAGAATGGAAAGTC, Rv: GATGCTGCTTACATGTCTCG), FOXP3 (Fw: TCAAGCACTGCCAGGCG, Rv: CAGGAGCCCTTGTCGGAT), RORγT (Fw: AAGACTCATCGCCAAAGCAT, Rv: TCCACATGCTGGCTACACA), GATA3 (Fw: CTGCAATGCCTGTGGGCTC, Rv: GACTGCAGGGACTCTCGCT) and T-bet(Fw: CCCCAAGGAATTGACAGTTG, Rv: GGGAAACTAAAGCTCACAAAC) were obtained from TIB Molbiol (Berlin, Germany). PCR reaction was performed with the LightCycler system from Roche, using SYBR Green I kit or SYBR Green FastStart kit from Roche. Ct values were compared to values obtained from mRNA samples isolated from tetanus toxoid stimulated PBMC.

2.3. Flow cytometry

After 6 days of culture, CD25 depleted PBMC were FACS stained with CD3-PE Cy7 (Biolegend, San Diego, CA, USA), CD4-APC (eBioscience, San Diego, CA, USA), FOXP3-PerCP Cy 5.5 (clone PCH101, eBioscience), RORγT-PE (eBioscience), T-bet-FITC (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and GATA3-PE (BD Bioscience). In other experiments, after 6 days of culture, cells were stimulated with phorbol 12-myristate 13-acetate (PMA, 5 ng/ml) and Ionomycin (1 μg/ml) in the presence of monensin (Golgistop, BD Biosciences) for 4 h. Subsequently cells were intracellular FACS stained for IL-10-APC (Biolegend), IL-17a-Alexa Fluor 488 (eBioscience), IFNγ-PE (BD Bioscience) or IL-4-PE (BD Bioscience).

Cells were analysed using a FACS Canto with FACS Diva software (BD Biosciences).

2.4. Statistics

Statistical significance was tested using Prism 4.0 for Macintosh with a Kruskal–Wallis test. $p \leq 0.05$ was considered significant. Dunn's multiple comparisons test was used to identify significant differences between strains. Correlations between experiments were tested with Spearman's rank correlation.

3. Results

In order to investigate the immune modulatory properties of strains with a high throughput method, PBMC from healthy volunteers were co-cultured with the strains in a 1:10 ratio. After 6 days of culture, CD4$^+$ T cells were isolated and lysed. Subsequently, the expression of transcription factors specific for CD4$^+$ T cell subsets was investigated by real time PCR. FOXP3, T-bet, GATA3, and RORγT are thought to be specific for Treg, TH1, TH2 and TH17 cells respectively.[16] The expression of the transcription factor mRNA of co-cultured PBMC was subsequently compared to the expression in the absence of bacteria in the culture medium. As shown in Fig. 1a–d, many differences between strains were observed. Some strains seem to activate different subtypes of T helper cells (e.g. *L. salivarius*, FOXP3 and RORγT, Fig. 1a, c), while others specifically induce one subtype (e.g. *L. lactis* W19, T-bet, Fig. 1b). Although the differences between the strains were significant for all individual transcription factors (Kruskal–Wallis,

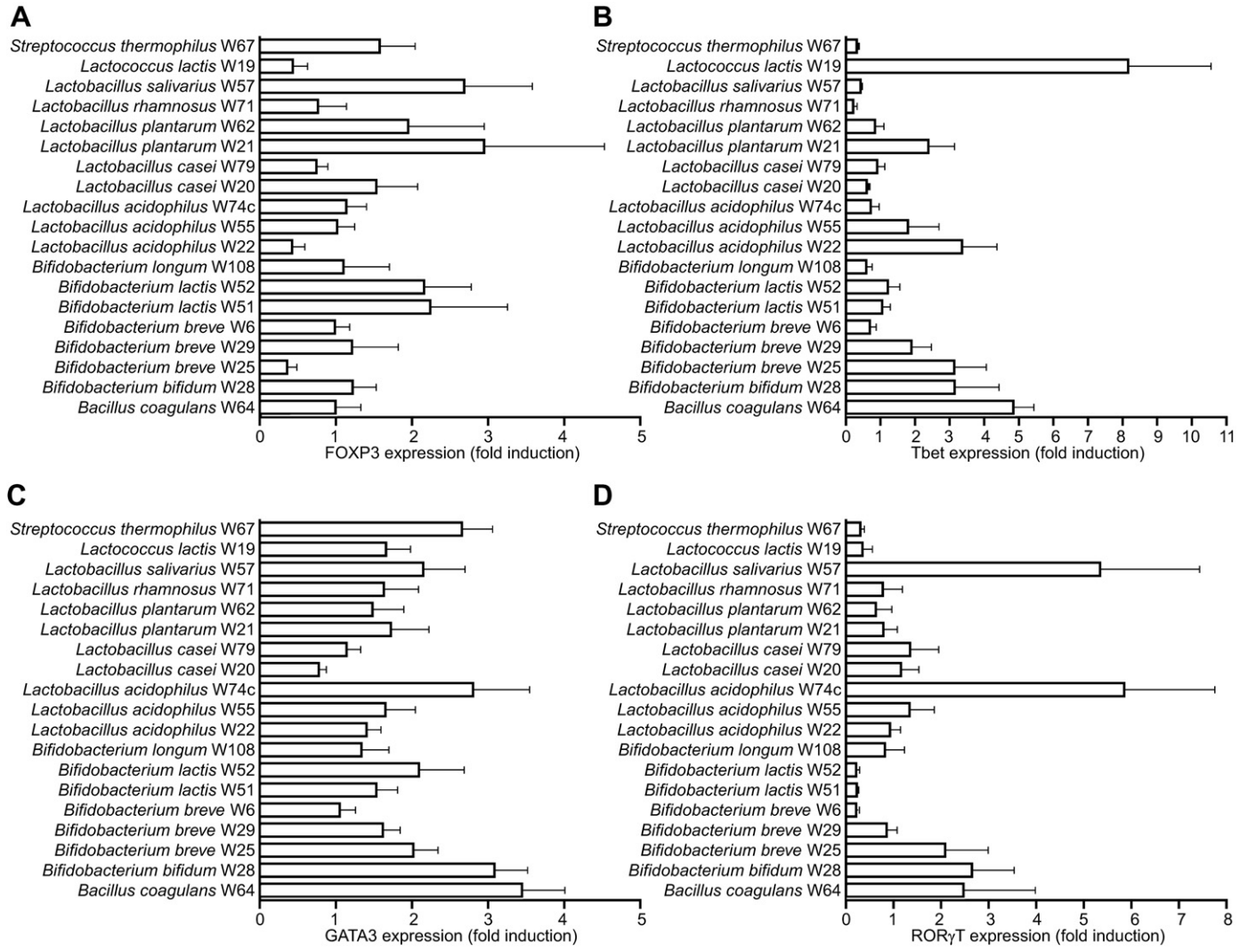

Fig. 1. Transcription factor mRNA in $CD4^+$ T cells after culture. Fold induction of the transcription of mRNA of FOXP3 for Treg (A), T-bet for TH1 (B), GATA3 for TH2 (C), and RORγT for TH17 (D) of CD4 T cells in PBMC co-cultured with probiotic strains compared to cells cultured in medium alone. CD4 cells were isolated from PBMC after 6 days of culture, lysed, and the transcription of specific mRNA compared to β2M mRNA transcription by rtPCR. Differences between strains in transcription of mRNA were significant for all genes tested ($P \leq 0.01$, Kruskal–Wallis), $n = 5$.

$P \leq 0.01$), the magnitude of the differences varied. The differences in GATA3 expression were less pronounced than the differences in for example T-bet and RORγT mRNA expression. No post-hoc tests were performed as the Bonferroni correction with these many strains and comparisons would reduce the probability of finding significant differences.

mRNA expression of the transcription factors defining the CD4 T cells subsets is a good indication for the fate of the T cells. However, mRNA does not necessarily lead to stable protein expression. In order to show that different T cell subsets are actually induced in the co-cultures, we subsequently measured transcription factor protein expression by intracellular FACS staining with a smaller number of strains. For these experiments we selected strains that showed an interesting profile in the expression of mRNA: *L. plantarum* W21 and *B. lactis* W52 for FOXP3 mRNA induction, *S. thermophilus* W67 for GATA3, *L. lactis* W19 T-bet, *L. salivarius* W57 both FOXP3 and RORγT. CD25 depleted PBMC were co-cultured with these strains for 6 days. At day 6 FACS staining was performed.

Figure 2a–d shows the percentages of $CD3^+CD4^+$ T cells positive for the protein of the specific transcription factor after culture with the strains mentioned. Most of the strains that induced mRNA of a specific transcription factor also induced expression of the protein. The strains *L. plantarum* W21 and *L. salivarius* that induced FOXP3 mRNA also induced high percentages of $FOXP3^+$ CD4 cells. *L. lactis* W19 and *L. salivarius* W57 also showed a similar pattern in mRNA and protein expression for T-bet and RORγT expression respectively. On top of that, significant differences between strains were observed in the induction of specific T cell subsets. *L. salivarius* W57 was shown to be the most potent inducer of $FOXP3^+$ cells, whereas *L. lactis* W19 induced the most T-bet^+ cells. These two strains induced the most $ROR\gamma T^+$ cells as well.

Most $CD4^+$ T cells produce cytokines in order to perform their biological function. For each T helper cell subset, the profile of cytokines produced is different (IL-10 for cells with a regulatory phenotype, IFNγ, IL-4 and IL-17 for TH1, TH2, TH17 cells respectively). To show that the T cells induced in our previous experiments really produce their specific effector molecules, we again co-cultured CD25 depleted PBMC with the selected strains and performed FACS staining after 4 h incubation with PMA/Ionomycin and monensin. Figure 4a–d shows that most cytokine profiles are comparable with the profiles of transcription factor mRNA and protein. Specifically, the strains that induced protein expression of

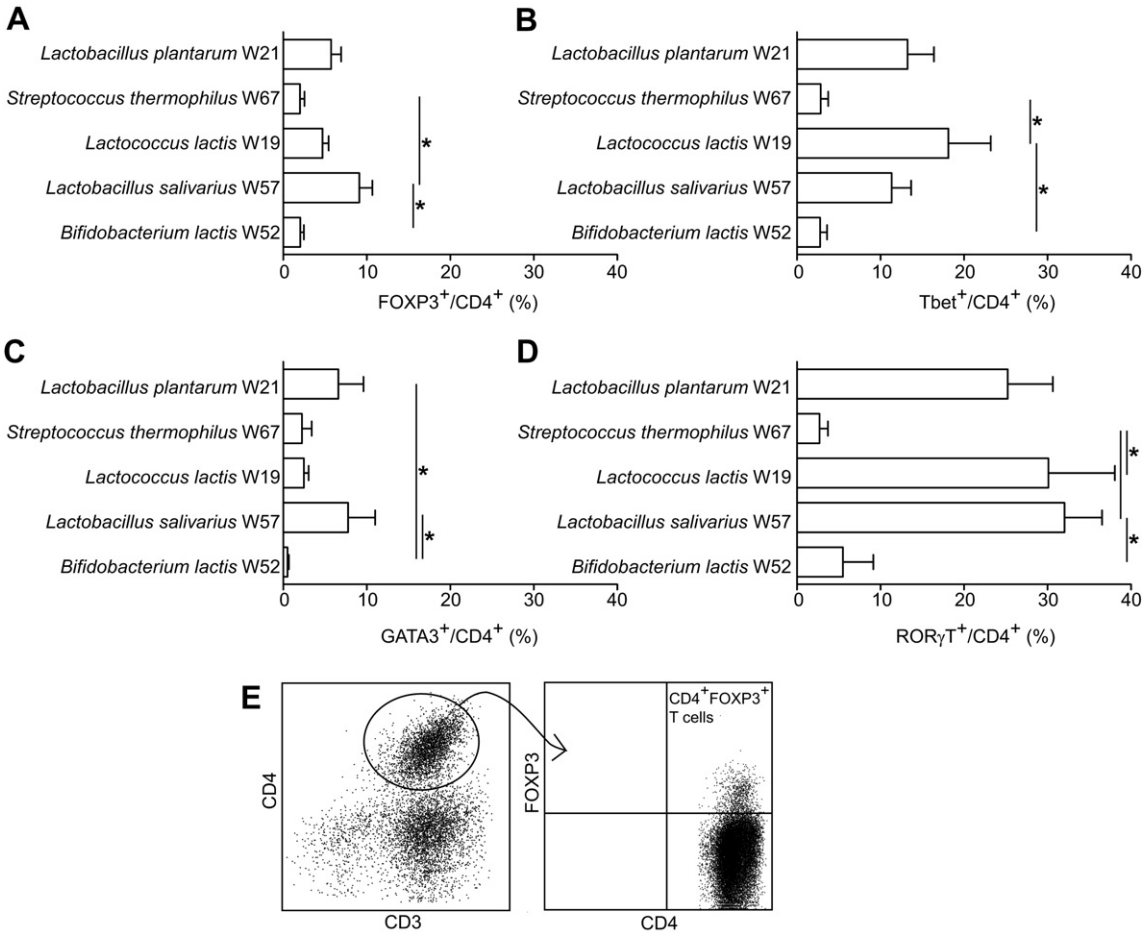

Fig. 2. Transcription factor protein in $CD4^+$ T cells after culture. Percentage of $FOXP3^+$ (A), T-bet^+ (B), $GATA3^+$ (C), and $ROR\gamma T^+$ (D) $CD3^+CD4^+$ T cells. After 6 days of culture of CD25 depleted PBMC, cells were FACS stained for transcription factors. *, significant difference according to Dunn's multiple comparisons test. Gating strategy is shown in E. $n = 6$.

the transcription factors also induced specific cytokine production. This is nicely shown for T-bet expression and IFNγ production, and for RORγT and IL-17 production. The strains that significantly induced the highest percentage of T-bet^+ and $ROR\gamma T^+$ cells, also did so for the corresponding cytokines. GATA3 expression is less in accordance with IL-4 production. Finally, *L. plantarum* W21 and *L. salivarius* W57 induce both $FOXP3^+$ cells and IL-10 producing CD4 T cells although there was no significant difference found for the IL-10 producing cells.

In order to show that our methods are consistent with each other, we performed a Spearman's rank correlation test with the strains that were tested with all methods. We analysed the correlation between the percentage of cytokine producing cells and the upregulation of mRNA for the specific transcription factor or the percentage of transcription factor positive cells measured by FACS (Fig. 4). Although for most correlations significance was not reached, the methods used to test the induction of Treg, TH1 and TH17 cells all have a positive relation (Spearman $\rho > 0$). The measurements on the induction of TH2 cells however also showed a negative correlation (Spearman $\rho < 0$) between GATA3 mRNA and IL-4 producing cell percentage, indicating that our methods were not consistent for this cell type.

As shown in Fig. 4a, we observed a considerable inter-donor variation with most techniques used. Therefore, we constructed a heat map to find out whether most donors had a comparable pattern of reaction towards the different strains (Fig. 4b). Indeed, some donors react more or different than others. However, most donors show consistent reaction towards the strains, forming clusters of differentiation towards a certain T helper subtype. This was observed for all strains and techniques used.

4. Discussion

Strains of human gut derived lactic acid bacteria have potent capacities to influence the immune system when used as a supplement. Each strain seems to be unique in the subtypes of CD4 T cells that it promotes. We and others already showed this for Treg induction[15] and for the production of cytokines.[17] We now extend these observations by showing that lactic acid bacteria significantly differ in their capacity to promote TH1 cells, but also TH2, Treg and TH17 cells. This finding adds important information to the concept of probiotic use as a food supplement for the prevention or treatment of immune mediated diseases such as allergies and auto-immune diseases. Our data suggest that not any strain of probiotic bacteria is able to mount a favourable immune response for any disease and that the strain used can make an important difference for the outcome of clinical experiments. For example, it is likely that the inconsistencies in the outcome of studies such as on the prevention of atopic disorders is due to subtle differences in the strains used.[18–21] Studies conducted with the same strain have more consistent outcome.[22]

As it is important to monitor the immune influencing capacities of strains, a reliable high throughput method is a helpful tool to screen for wanted and unwanted effects of probiotics. Co-culture of strains with PBMC and subsequent transcription factor mRNA analyses seems suitable for this purpose. The activation or

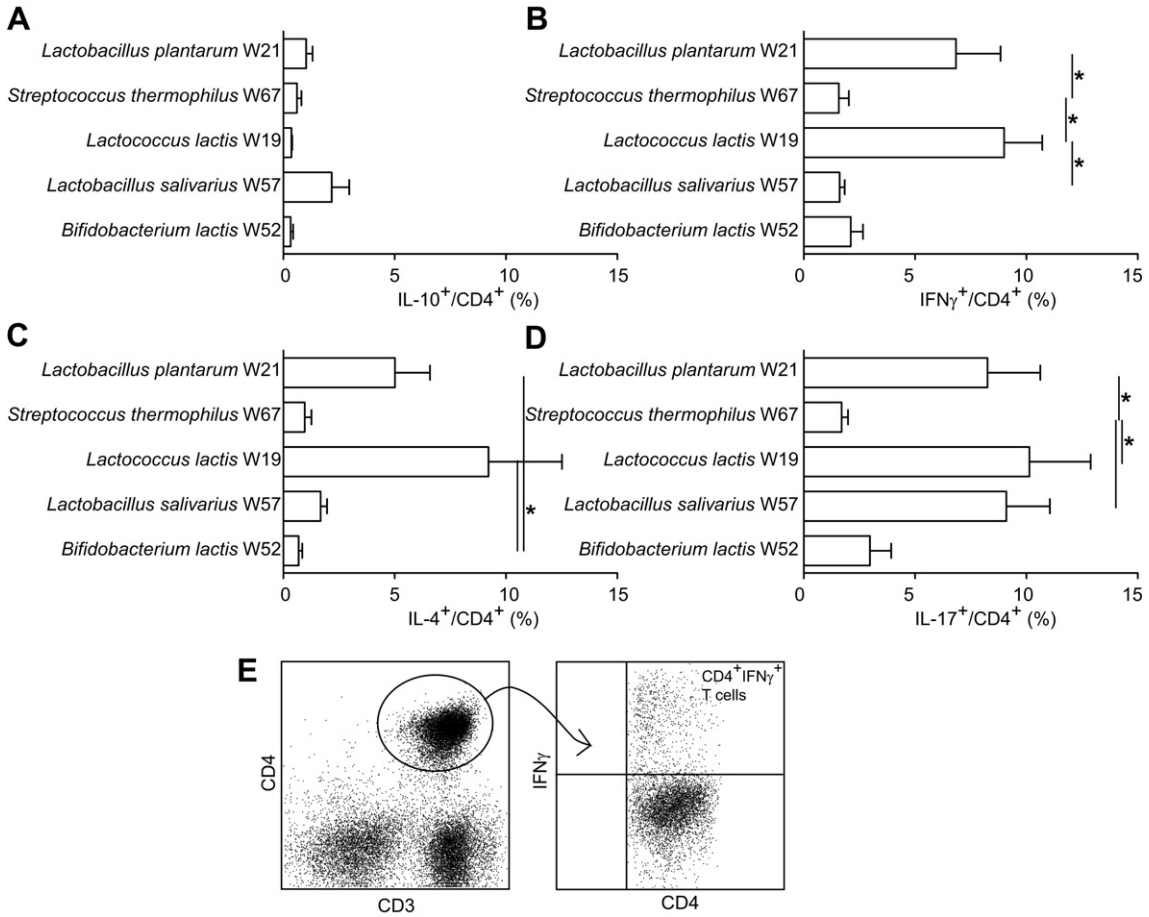

Fig. 3. Cytokine production by $CD4^+$ T cells after culture. Percentage of IL-10^+ (A), IFNγ^+ (B), IL-4^+ (C) and IL-17^+ (D) $CD3^+CD4^+$ T cells. After 6 days of culture of CD25 depleted PBMC, cells were restimulated with PMA/Ionomycin for 4 h in the presence of monensin. Cells were subsequently FACS stained for intracellular cytokines. *, significant difference according to Dunn's multiple comparisons test. Gating strategy is shown in E. $n = 6$.

induction of T cell subtypes was shown consistently on several levels; the transcription factor mRNA content, transcription factor protein as well as cytokine production. These methods correlated well for most T cell subsets, making all methods used in this research possible candidates for high throughput measurements of large numbers of strains, depending on a laboratory's possibilities.

Most *in vitro* analysis of immune modulating properties of bacterial strains are performed by measurements on cytokines in the supernatants of PBMC or other cell cultures in the presence of bacteria.[17,23] Up- or downregulation of surface markers on dendritic cells is also a common approach.[13] Although these methods definitely have benefits (broad scan of immune responses), the response of the adaptive immune system needs to be addressed as well. T cells are likely candidates for local immune activity outside the gut making these cells the targets in settings as for example eczema and asthma. Analysis of T cell responses as described here can perfectly be combined with analysis of cytokines in the supernatant of the same culture, increasing the value and reliability of the screening.

Because of high variability between donors, and the use of different donors for each experiment, the correlations were not significant for all tests. Nevertheless, the correlation was shown to be positive for Treg, TH1 and TH17 cell induction, indicating that the strains that induce mRNA of a specific T cell type will also induce a high percentage of specific cytokine producing cells. Our methods showed not to be suitable for the measurement of TH2 cell induction by probiotics. This can be due to the fact that GATA3 is known to be necessary for more cellular processes than TH2 differentiation,[24] or timing of the measurements. Of course, each mRNA, and protein has its own kinetics, making it hard to measure all processes with a few tests on a few time points. Possibly the timing for TH2 mRNA and proteins was suboptimal.

Although strains have a clear tendency to induce either subtype of T helper cell, this effect is not shown with identical strength for all donors tested, resulting in considerable variation in our results. This finding, as depicted in Fig. 4b, adds another layer to the complex field of immune modulation by probiotics. Possibly the variation in the human population in reactivity towards a certain bacterial strain warrants a personalized approach. Before supplementing a patient with a certain strain, the quality and quantity of the patients T cell response towards this strain can be determined with either of the described methods. On the other hand, supplementation with a probiotic mixture (e.g. a multi-species probiotics) of strains with a certain immune modulating capacity might be beneficial for a broader group of individuals.[25] *In vitro* analysis of T cell responses towards such a mixture would be of great interest.

Whether the induction of different T cell subsets is also consistent *in vivo* and whether these cells are able to influence immune mediated diseases still remains to be tested. It has been shown by several authors that *in vitro* experiments do not necessarily correlate with clinical outcome.[26,27] Nevertheless, application of probiotic strains without proper knowledge on their immunological capacities can also have unwanted effects.[28] *In vitro* research using human PBMC cultures can give a good indication, although this does not perfectly mimic the *in vivo* situation. Both *in vitro* and *in vivo* parameters should be selected carefully in order to find and correlate effects of probiotics. Testing the strains used here in

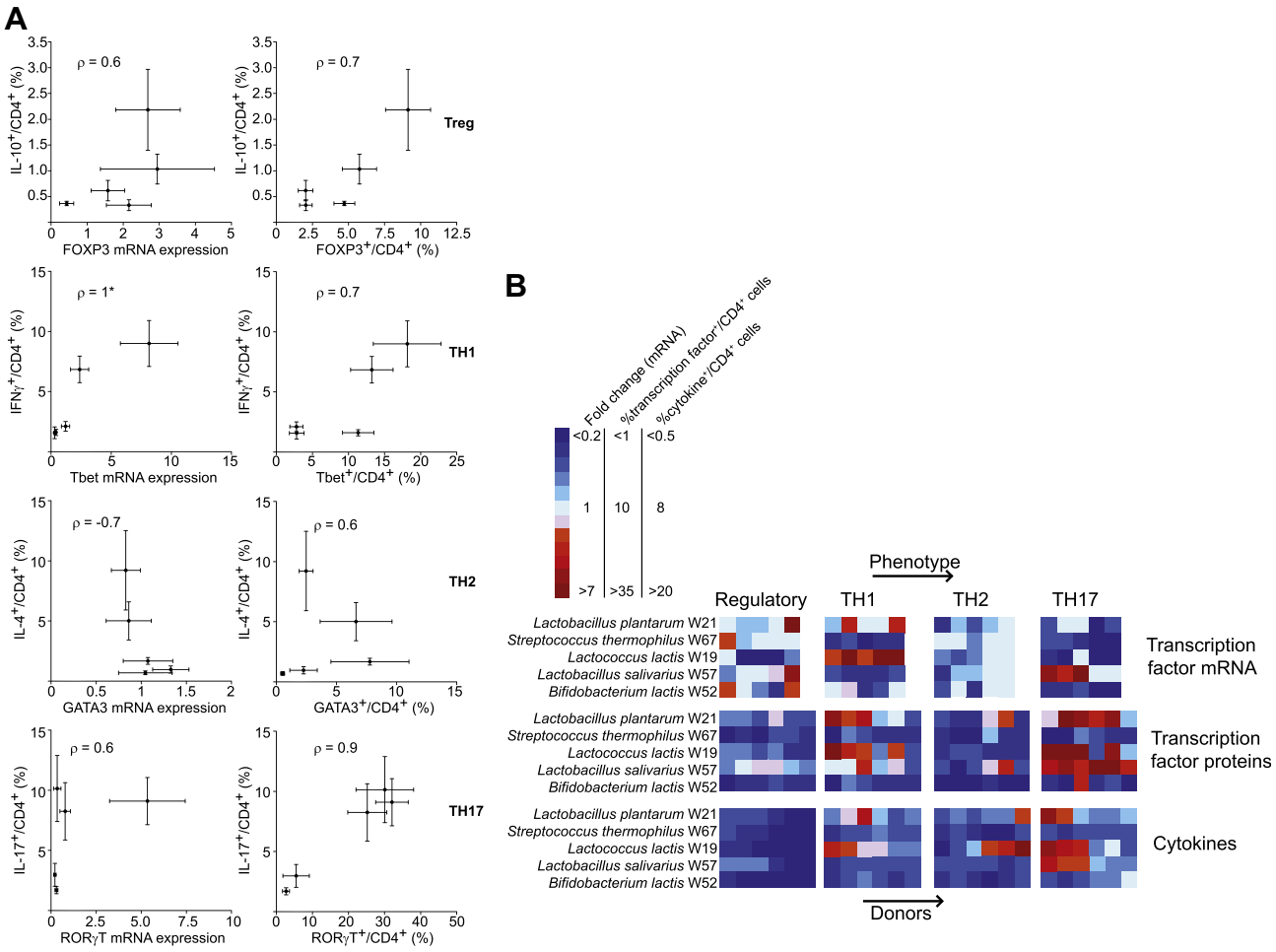

Fig. 4. Analysis of correlations and donor variability. Correlation between expression of transcription factor mRNA and percentage cytokine producing cells (A, left part) and percentage of transcription factor positive cells and percentage cytokine producing cells (A, right part). Spearman ρ is given as a measure of correlation. *, significant correlation according to Spearman's signed rank test. $n = 5$ for mRNA data and $n = 6$ for FACS data of transcription factor expression and cytokine production. Heat map (B) indicating the responses of all donors towards the strains used measured by transcription factor mRNA PCR (FOXP3, T-bet, GATA3, RORγT respectively), transcription factor protein FACS (FOXP3, T-bet, GATA3, RORγT respectively) or cytokine FACS (IL-10, IFNγ, IL-4, IL-17 respectively) as depicted in the Figs. 1–3. The legend indicates the values corresponding to the colours for each technique. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

several experimental models including colitis and atopic dermatitis models, and comparing the results to our *in vitro* data would be of great interest. Treg inducing strains will likely have a beneficial effect on these diseases, whereas TH1, TH17 or TH2 inducing strains might aggravate disease.

There is still much to learn regarding the determinants of the diverse immune responses elicited by different bacterial strains. The identification of closely related strains that differ in the type of immune response they elicit is of special interest. In our study *L. acidophilus* W74c and *L. acidophilus* W22 showed remarkable differences in their capacity to induce RORγT. Further screening of the genomes of these strains using micro-array might give more insight in the specific gene(s) responsible for the immune modulation. That the type of immune response induced by gut derived bacteria can depend on one single molecule has elegantly been shown by a study of Mazmanian et al.[29] In this study it was demonstrated that *Bacteroides fragilis* depends on polysaccharide A (PSA) to protect against inflammation in an experimental colitis model and that PSA is sufficient for the protective effect. Also, the capacity of strains to ligate certain receptors expressed by the innate immune system can differentiate between activation of the immune system. Attachment to DC-SIGN expressed by DC has been shown to prime DC to induce FOXP3$^+$ regulatory T cells.[30] The differences we observe in *in vitro* immune modulation between strains might also be partially explained by ligation of DC-SIGN or other receptors.

We conclude that to make well-founded choices on the type of probiotic strains to be used for clinical application, *in vitro* monitoring of the immunological effects of the strains using a high throughput method is highly recommendable.[15] By building on our current knowledge of strain specific immune modulatory effects it may become possible to design clinically effective, bacteria based strategies to maintain and promote health.

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Statement of authorship

SdR, **IMdK** and **GTR** designed research, **MvE** and **SdR** conducted the research and analysed the data, **SdR** and **IMdK** wrote the paper, **MOH** and **BJP** had primary responsibility for final content.

Conflict of interest

The authors declare no conflict of interest.

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