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RESEARCH ARTICLE

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Mucosal-associated invariant T-Cell (MAIT) activation is altered by chlorpyrifos- and glyphosate-treated commensal gut bacteria

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ABSTRACT

Mucosal-associated invariant T-cells (MAIT) can react to metabolites of the vitamins riboflavin and folate which are produced by the human gut microbiota. Since several studies showed that the pesticide chlorpyrifos (CPF) and glyphosate (GLP) can impair the gut microbiota, the present study was undertaken to investigate the impact of CPF and GLP treatment on the metabolism of gut microbiota and the resulting bacteria-mediated modulation of MAIT cell activity. Here, *Bifidobacterium adolescentis* (*B. adolescentis*), *Lactobacillus reuteri* (*L. reuteri*), and *Escherichia coli* (*E. coli*) were treated with CPF (50–200 µM) or GLP (75–300 mg/L) and then used in MAIT cell stimulation assays as well as in vitamin and proteome analyses. All three bacteria were nonpathogenic and chosen as representatives of a healthy human gut microflora. The results showed that *E. coli* activated MAIT cells whereas *B. adolescentis* and *L. reuteri* inhibited MAIT cell activation. CPF treatment significantly increased *E. coli*-mediated MAIT cell activation. Treatment of *B. adolescentis* and *L. reuteri* with CPF and GLP weakened the inhibition of MAIT cell activation. Riboflavin and folate production by the test bacteria was influenced by CPF treatment, whereas GLP had only minor effects. Proteomic analysis of CPF-treated *E. coli* revealed changes in the riboflavin and folate biosynthesis pathways. The findings here suggest that the metabolism of the analyzed bacteria could be altered by exposure to CPF and GLP, leading to an increased pro-inflammatory immune response.

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MAIT cells; gut microbiota; pesticides; glyphosate; chlorpyrifos

Introduction

Commensal bacteria in the human gut provide important functions for the host including protection against pathogens, nutrient digestion, production of metabolites as well as the modulation of host immune responses (Sekirov et al. 2010). Furthermore, microbiota of the gut have also been recognized as a source of vitamins which cannot be synthesized by the human body. Amongst these, riboflavin and folate (Vitamins B_2 and B_9) have been shown to be produced by probiotic bacteria in the human gut, as well as by food-associated lactic acid bacteria (Hill 1997; Pompei et al. 2007; LeBlanc, et al. 2011, 2013). For example, *Bifidobacterium adolescentis* (*B. adolescentis*), *Lactobacillus reuteri* (*L. reuteri*), and *Escherichia coli* (*E. coli*) have each been shown to contribute to folate production (Santos et al. 2008; LeBlanc et al. 2011; Rossi et al. 2011; Thiaville et al. 2016). This vitamin is implicated in DNA replication, repair, and methylation, as well as in amino acid biosynthesis (LeBlanc et al. 2013). Besides folate, riboflavin production has been described in a number of strains of *L. reuteri* and *E. coli* (Fischer et al. 1996; Wang et al. 2015; Thakur et al. 2016). Riboflavin is essential as it is the precursor of the co-enzymes flavin mononucleotide and flavin adenine dinucleotide – both of which are both involved in redox reactions (LeBlanc et al. 2013).

Recent studies have shown that metabolites of riboflavin and folate can regulate the activity of a novel innate-like T cell subtype, the mucosal-associated invariant T (MAIT)-cells. The latter is a T-cell type that is highly abundant in human blood and mucosal tissues like the liver and gut (Kjer-Nielsen et al. 2012; Reantragoon et al. 2013). MAIT cells are characterized by a semi-invariant T-cell receptor (TCR) (Vα7.2 in humans) restricted to a non-classical MHC Class I-related (MR1) molecule and by expression of CD161 (Reantragoon et al. 2013). Precursors of bacterial riboflavin biosynthesis can activate MAIT cells. In contrast, folate metabolites inhibit MAIT cell activity by covalently binding to MR1, thereby blocking cell activation by riboflavin precursors (Eckle et al. 2014; Kjer-Nielsen et al. 2018). After activation, MAIT cells produce pro-inflammatory cytokines (i.e. tumor necrosis factor [TNF]-α and interferon [IFN]-γ) and cytolytic proteins (i.e. perforin and granzyme B) (Chiba et al. 2018; Rudak et al. 2018).

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Supplemental data for this article can be accessed here.

An imbalance between activating and inhibiting metabolites may impact the function of MAIT cells. This in turn could lead to chronic activation of these cells and as a consequence, to increased production of inflammatory cytokines. Indeed, MAIT cells have been found in the inflamed tissues of patients with Crohn's disease, multiple sclerosis, rheumatoid arthritis and asthma (Serriari et al. 2014; Carolan et al. 2015; Chiba et al. 2018; Lezmi and Leite-de-Moraes 2018). Likewise, a disturbance of the gut microbiota (dysbiosis) is involved in the same pathologies (Serriari et al. 2014). This leads to the assumption that bacterial homeostasis is linked to immune homeostasis, and although not exclusively, it might be reflected by a modulation in MAIT cell activity.

Environmental contaminants, including pesticides that can reach the human gut via food ingestion might potentially impact on gut homeostasis. Chlorpyrifos (CPF) is one of the most frequently used broad-spectrum insecticides. It is used to control a multitude of insects during the cultivation of grains, cotton, vegetable crops, lawns, and ornamental plants (Thengodkar and Sivakami 2010) and is also used in some instances in veterinary medicine (Marquez Giron et al. 2017). It was recently shown that CPF causes gut microbiota dysbiosis in mice, rats and zebrafish (Zhao et al. 2016; Fang et al. 2018; Wang et al. 2019). Glyphosate (GLP) is a commonly used broad-spectrum herbicide that is applied globally for weed control (Green 2018; van Bruggen et al. 2018). GLP and the GLP-based herbicide Roundup were shown to be able to perturb the gut microbiota of honey bees, mice, and rats (Aitbali et al. 2018; Mao et al. 2018; Motta et al. 2018); however, other studies have shown no effect (Nielsen et al. 2018). There is still an ongoing debate about the effects of glyphosate on the microbiota. Due to this combination of frequent usage and possible effects on microbiota, the present study was undertaken to investigate the potential impact of the pesticides CPF and GLP on select commensal bacteria.

Recently, it was shown that the microenvironment can impact the bacterial metabolism leading to a modification of MAIT cell activation (Schmaler et al. 2018). Thus, the hypothesis here was that pesticide-induced changes in bacterial metabolism might alter the riboflavin and/or folate production by these microorganisms, thereby impacting the activation of MAIT cells. In the current study, the bacterial strains *L. reuteri*, *B. adolescentis* and *E. coli* were investigated as their health-promoting effects are well described (Malchow 1997; Kruis et al. 2004; Lee et al. 2008; Khokhlova et al. 2012; Wu et al. 2017; Mu et al. 2018), as have been their capacity to produce riboflavin and folate.

In the context of MAIT cells, *E. coli* has been described as being able to activate MAIT cells whereas *L. reuteri* has an inhibitory effect (Dias et al. 2016; Johansson et al. 2016; Schmaler et al. 2018). With regard to *B. adolescentis*, there is no data currently available regarding MAIT cell activation or inhibition. However, due to its already-described folate-producing activities, it was assumed here that this strain might inhibit MAIT cell activation. Thus, the aim of the present study was to evaluate whether MAIT cell-activating bacteria as well as MAIT cell-inhibiting bacteria might be affected by CPF or GLP treatment.

Materials and methods

Stock solutions

Chlorpyrifos (CPF) (Cheminova, Lemvig, Denmark) was dissolved in DMSO (Appli-Chem, Darmstadt, Germany) as a 200 mM stock solution and then stored at room temperature. Commercially-available glyphosate (GLP; in the formulation Roundup LB Plus; Monsanto Agrar Germany, Düsseldorf, Germany) containing 360 g GLP/L was diluted in bacteria culture medium and stored at 4 °C. Acetyl-6-formylpterin (Ac-6-FP, Schircks Laboratories, Bauma, Switzerland) was dissolved in 17 mM NaOH to make a 5 mM stock solution and then stored at −20 °C.

Bacteria preparation

Escherichia coli DH5α (ThermoFisher Scientific, Waltham, MA, USA) was cultured for 16 hr in LB medium Miller (Carl Roth GmbH, Karlsruhe, Germany) at 37 °C with shaking at 175 rpm. The starter culture was then diluted 1:50 with fresh LB medium Miller. Both *Bifidobacterium adolescentis* E298b Variant c (DSM-20086) and *Lactobacillus reuteri* M6220-5A (DSM-28673) (both from DSMZ, Braunschweig, Germany) were cultured in Bifidobacteria medium and MRS medium, respectively (Supplementary Table S1) under anaerobic conditions for 3 d (pre-culture) at 37 °C and 175 rpm, then diluted 1:50 in fresh culture medium and grown for an additional 48 hr. After this culture time (16 hr for *E. coli* and 48 hr for *B. adolescentis* and *L. reuteri*) all bacterial strains were treated for 16 hr with CPF (at final concentrations of 50, 100, or 200 µM), GLP (final concentrations of 75, 150, or 300 mg/L), or the respective solvents without CPF or GLP. The bacteria were then fixed at room temperature in 1% Formaldehyde (ThermoFisher) for 10 min (*E. coli*) or 5 min (*B. adolescentis* and *L. reuteri*). After washing three times with phosphate-buffered saline (PBS; pH 7.4), the bacteria were counted using a Multisizer 3 Coulter Counter (Beckman Coulter, Indianapolis, IN) and also frozen as aliquots at −80 °C (Supplementary Table S1). Identically-treated bacteria from three independent preparations were pooled to avoid batch effects and used for the MAIT cell stimulation assays (see below).

Delayed growth toxicity assay for bacterial viability

Bacteria were treated with CPF or GLP as above. In these studies, 0.4 M NaOH was used as a positive control for inducing bacterial cell death. At the end of the 16-hr period, the now-treated bacteria were diluted in fresh bacteria culture medium and then grown an additional 6 hr (*E. coli*) or 24 hr (*B. adolescentis* and *L. reuteri*). Optical density of the culture suspensions was then measured at 600 nm in a UV-1800 Spectrophotometer (Shimadzu, Kyoto, Japan).

MAIT cell activation assays

Pseudonymous buffy coat samples from six healthy volunteers were obtained from the blood bank at the University of Leipzig. All participants had given written informed consent. The study was approved by the Ethics Committee of the University of Leipzig (Ref. #079-15-09032015). Peripheral blood mononuclear cells (PBMC) were isolated from the buffy coats by density-gradient centrifugation using Ficoll-Paque Plus (GE Healthcare, Little Chalfont, UK). Cells were stored in liquid nitrogen until needed in the stimulation assays.

For the assay, PBMC were cultured in IMDM (GlutaMax supplement, Fisher Scientific, Schwerte, Germany) supplemented with 10% fetal bovine serum (FBS, Biochrom, Berlin, Germany),

1X Pen-Strep Solution (Biowest, Nuaillé, France), and 50 µM β-mercaptoethanol (AppliChem). The cells were plated at 10^6 cells/well in U-bottom 96-well microplates (Greiner Bio-One, Frickenhausen, Germany). After incubation overnight at 37 °C in a 5% CO_2 incubator, the cells were stimulated for 6 hr with bacteria. Brefeldin A (10 µg/mL) (Sigma, St. Louis, MO) was added to all wells for the final 4 hr of this culture period.

Prior experiments determined the optimal bacterial concentration of E. coli for MAIT cell activation. These assays showed that 25–50 bacteria (*E. coli*) per cell) led to a clear MAIT cell activation (Supplementary Figure S1). For all future experiments we chose 30 bacteria (*E. coli*) per cell (BpC). This level enabled increases and decreases of activation by pesticide-treated *E. coli* to be demonstrated (data not shown). Accordingly, a concentration of 30 BpC *E. coli* was used in the present MAIT cell activation studies. On the other hand, due to a general lack of information about MAIT cell-activating capacities for *B. adolescentis* and *L. reuteri*, a higher concentration of 100 BpC of each strain was used here to provoke MAIT cell activation.

To visualize any inhibition of MAIT cell activation, PBMC were seeded as above. Prior to the assays, titration experiments were conducted to determine the appropriate amount of *B. adolescentis* and *L. reuteri* to achieve inhibition of MAIT cell activation of ≈50% (Supplementary Figure S2). A level of 50% inhibition enable detection of either an increase of inhibition by pesticide-treated *B. adolescentis* and *L. reuteri* (i.e. leading to lower cytokine production) or a decrease of inhibition (i.e. leading to higher cytokine production). In this assay, the PBMC were pre-stimulated with untreated or CPF- or GLP-treated *B. adolescentis* (20 BpC) or *L. reuteri* (6 BpC) for 1 hr or left untreated (i.e. without bacteria). As a positive control for inhibition of MAIT cell activation, PBMC were pre-stimulated with Ac-6-FP (50 µM) for 1 hr. Thereafter, to all wells, untreated *E. coli* (30 BpC) was added and the cells were cultured for 6 hr. As above, 10 µg/mL Brefeldin A was added to all wells for the final 4 hr of the incubation period.

Antibody staining and flow cytometry

After *in vitro* stimulation, PBMC were stained with Fixable Viability Dye eFluor™ 506 (eBioscience, Frankfurt/Main, Germany) for dead cell exclusion, followed by cell surface staining for 30 min at room temperature (Supplementary Table S2). Thereafter, the cells were fixed in FACS™ lysing Solution (BD) and permeabilized using FACS™ Permeabilizing Solution 2 (BD Biosciences, San Jose, CA). Finally, the cells were intracellularly stained for 30 min at room temperature (Supplementary Table S2), and then analyzed in a BD FACSCanto™ II cytometer with FACS Diva software version 8.0.1 (BD Biosciences, San Jose, CA, USA). Flow cytometry data were analyzed with FlowJo Version 10.2 (FlowJo, Ashland, OR). A minimum of 150,000 viable T-cells/sample was acquired. The lymphocytes among the human PBMC were identified by means of FSC-A and SSC-A. MAIT cells were identified (as $CD3^+CD8a^+CD161^+TCRV\alpha7.2^+$) after exclusion of doublets and dead cells.

Note: $CD8a^+$ MAIT cells are termed "MAIT cells" hereafter for simplicity. TNFα and IFNγ production in these cells was also quantified (Supplementary Figure S3).

Riboflavin and folate analysis

To measure riboflavin and folate content, bacterial cell pellets of untreated and pesticide-treated bacteria ($n = 3$ independent experiment for each treatment) were lysed using CelLytic B Plus Kit (Sigma), according to manufacturer instructions - without adding the supplied Protease Inhibitor Cocktail. The riboflavin concentration was analyzed by LC-MS/MS whereas the total folate content was measured with the electrochemiluminescence immunoassay Elecsys Folate (Roche, Basel, Switzerland) (see Supplementary Methods).

Proteomic analysis

Samples of *E. coli* ($n = 6$) that had been untreated (solvent\vehicle) or treated with CPF (50–200 µM) were lysed using a Fastprep (FastPrep-24, MP Biomedicals, Eschwege, Germany). The proteome was then analyzed by LC-MS/MS (see Supplementary Methods).

Statistical analysis

In vitro stimulation assays were performed with cells from six donors in three independ-ent experiments, and data were normalized to the cytokine production in untreated *E. coli*. Folate and riboflavin analysis was conducted three times; raw data and solvent control normalized data are presented. All data are presented as means ± SEM. A one-way analysis of variance (ANOVA) was used to analyze the effect of chemical treatment in the *in vitro* assays and in the vitamin analyses. All analyses were performed using Prism v.7.04 (GraphPad Software, San Diego, CA, USA). A p-value $< .05$ was considered statistically significant.

For the proteomic studies, CPF-treated *E. coli* pellets of six independent experiments were used. Transforming and normalization of the protein intensity data to label free quantification (LFQ) values was performed by an in-house written R script. Intensities were $\log_2$-transformed and normalized by the transformed median intensities of the sample multiplied by the median of the $\log_2$ transformed medians of all samples. LFQ values were correlated with the CPF concentration with PERMANOVA using the corrplot package from R. Exact n numbers for all experiments are indicated below the respective figures. A p-value $< .05$ was considered statistically significant.

Results

MAIT cell activation

In the present study, MAIT cells were stimulated with fixed *E. coli*, *B. adolescentis* or *L. reuteri* in single stimulation assays and activation then assessed via measures of intracellular TNFα und IFNγ production. Stimulation with *E. coli* led to an increased number of TNFα- and IFNγ-producing MAIT cells (Figures 1(A,B)). The frequencies of *E. coli*-induced TNFα- and IFNγ-producing MAIT cells averaged 32.0 [± 4.9]% and 9.8 [± 1.5]%, respectively; unstimulated MAIT cells did not produce TNFα and IFNγ. Stimulation with *B. adolescentis* and *L. reuteri* did not induce TNFα and IFNγ production by MAIT cells, even at high concentrations of 100 BpC.

Since *B. adolescentis* and *L. reuteri* did not induce MAIT cell activation, these bacteria were assessed for potentials to inhibit activation by *E. coli*. The appropriate amount of *B. adolescentis* and *L. reuteri* was determined in titration experiments to achieve an inhibition of ≈50%; this was obtained using 20 bacteria per cell (BpC) *B. adolescentis* and 6 BpC *L. reuteri* (Supplementary Figure S2). The results show that in the co-stimulation assays, *B. adolescentis* and *L. reuteri* both reduced *E. coli*-induced MAIT cell activation (Figure 2(A)). A co-presence of *B. adolescentis* led

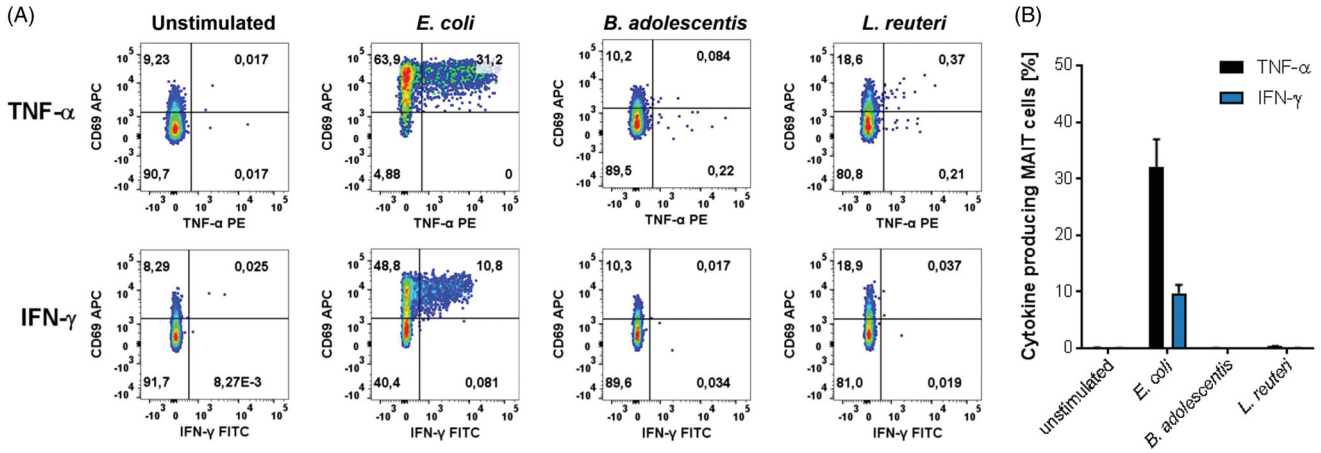

Figure 1. MAIT cell activation after single stimulation with different bacterial strains. PBMC were stimulated with fixed *Escherichia coli* (*E. coli*, 30 BpC), *Bifidobacterium adoles-centis* (*B. adolescentis*, 100 BpC) or *Lactobacillus reuteri* (*L. reuteri*, 100 BpC) for 6 hr. MAIT cells ($CD3^+CD8a^+CD161^+TCRV\alpha7.2^+$ populations) were detected by flow cytometry. Activation was measured via intracellular staining of TNFα and IFNγ production. Results are given as percentage of gated MAIT cell populations. (A) Representative dot-plots of bacteria-stimulated MAIT cells from $n = 6$ (unstimulated, *E. coli*) or $n = 2$ (*B. adolescentis*, *L. reuteri*) donors; summarized (mean ± SEM) in (B).

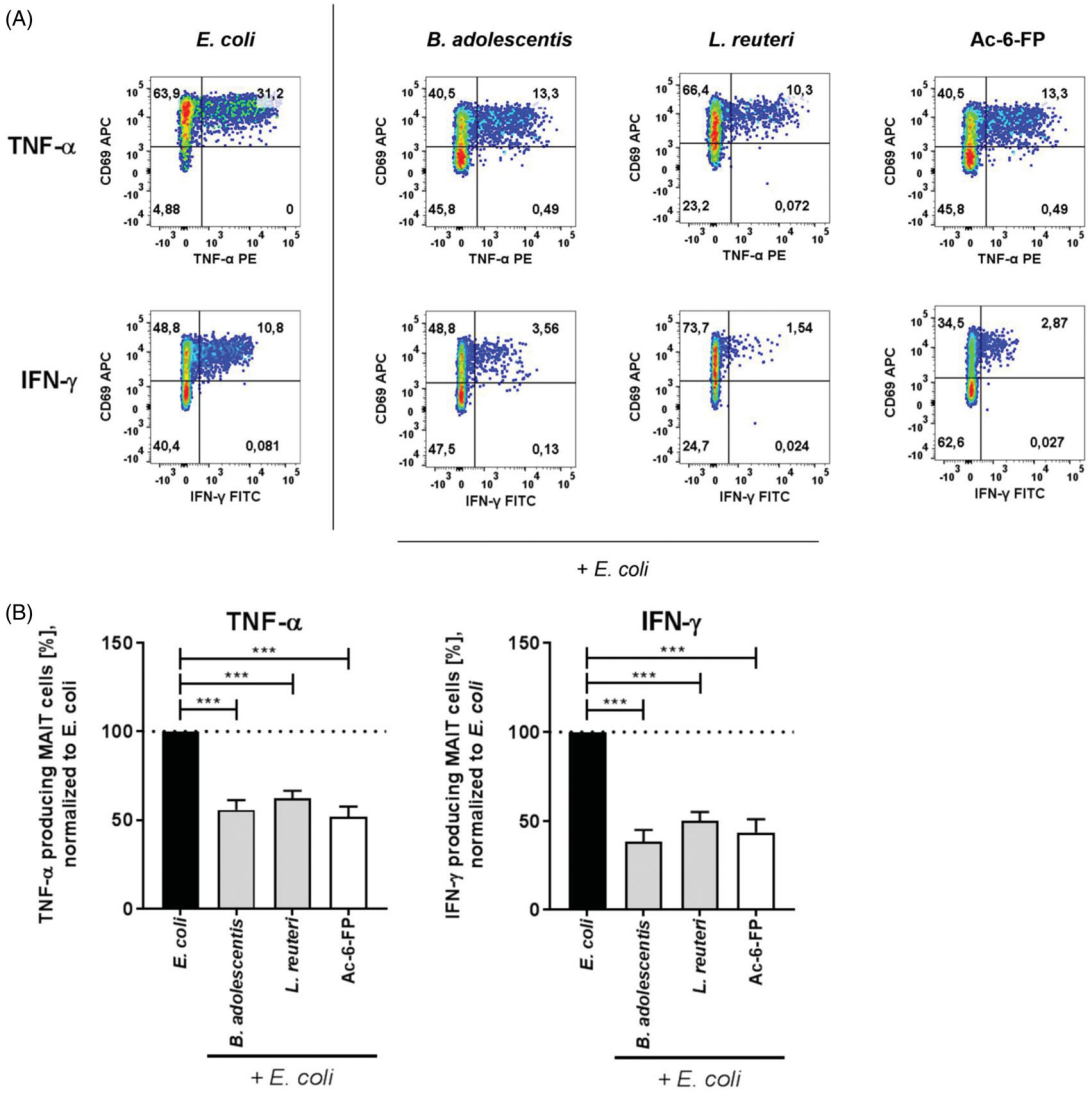

Figure 2. Inhibition of *E. coli*-induced MAIT cell activation by *B. adolescentis*, *L. reuteri*, or the folate metabolite acetyl-6-formylpterin (Ac-6-FP). PBMC were stimulated with fixed *E. coli* alone (30 BpC) or co-stimulated with fixed *E. coli* (30 BpC) and fixed *B. adolescentis* (20 BpC), *L. reuteri* (6 BpC) *or* Ac-6-FP (50 μM) for 6 hr. MAIT cells ($CD3^+CD8a^+CD161^+$ $TCRV\alpha7.2^+$ populations) were detected by flow cytometry. Activation of cells was measured via intracellular staining of TNFα and IFNγ production. Results given as percentage of gated MAIT cell population. (A) Representative dot-plots of bacteria and Ac-6-FP stimulated MAIT cells from $n = 6$ donors. (B) Summarized data after normalization to the single stimulation with *E. coli*. $N = 6$, mean ± SEM, one-way ANOVA. $^{***}p < .001$ vs. *E. coli*.

to 44.2% and 61.6% fewer TNFα- and IFNα-producing MAIT cells (compared to levels with *E. coli* alone), respectively (Figure 2(B)). Similar results were obtained in the *L. reuteri* co-stimulation assays (Figure 2(B)). The folate metabolite, Ac-6-FP (positive control for MAIT cell inhibition) also reduced *E. coli*-induced MAIT cell activation by nearly 50%.

Modulation of MAIT cell activity by pesticide-treated bacteria

To see whether pesticide-treated bacteria impacted activation\inhibition of MAIT cells, the bacterial strains were treated with CPF or GLP for 16 hr prior to fixation. Data from the growth assay revealed that neither CPF nor GLP had lethal effects on these bacterial strains at the levels used here (Supplementary Table S3). Subsequently, the pesticide-treated bacteria were used in MAIT cell stimulation assays and the numbers of cytokine-producing cells was then normalized to controls without CPF or GLP treatment. The data show that CPF treatment (200 µM) of *E. coli* induced a significantly higher number of TNFα ($p = .011$)- and IFNγ ($p = .002$)-producing MAIT cells compared to that by untreated *E. coli* controls (Figures 3(A,B)). GLP treatment (150 mg/L) of *E. coli* led to an increase in TNFα-producing MAIT cells ($p = .017$), but did not significantly alter IFNγ production by these cells.

As stimulation of MAIT cells with CPF- or GLP-treated *B. adolescentis* or *L. reuteri* did not induce IFNγ or TNFα production (data not shown), these CPF- or GLP-treated bacteria were tested in co-stimulation assays together with untreated *E. coli*. In all cases, the number of TNFα- and IFNγ-producing MAIT cells was normalized to the response induced by the untreated *E. coli* alone. The results showed that CPF-treatment of *B. adolescentis* reduced the extent of inhibition of MAIT cell activation compared to that by untreated *B. adolescentis* (Figure 4(B)). A similar pattern was observed with IFNγ production. In contrast, GLP treatment of *B. adolescentis* did not cause significant changes in impact on MAIT cell activation compared to that seen with untreated *B. adolescentis*.

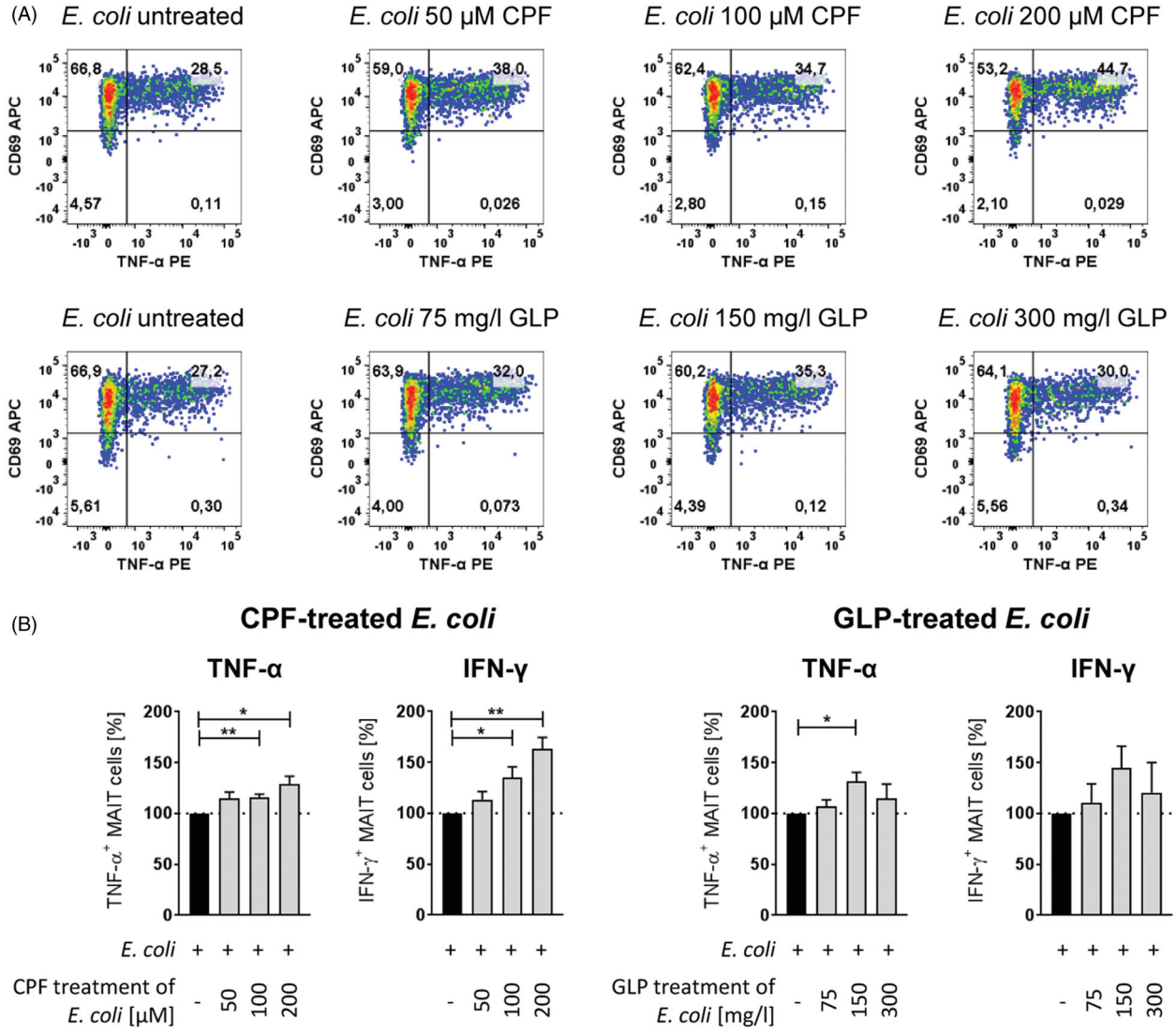

Figure 3. MAIT cell activation by chlorpyrifos (CPF)- or glyphosate (GLP)-treated *E. coli*. PBMC were stimulated with fixed CPF- and GLP- treated *E. coli* (30 BpC) for 6 hr. MAIT cells ($CD3^+CD8a^+CD161^+TCRV\alpha7.2^+$ populations) were detected by flow cytometry. Activation of cells was measured via intracellular staining of TNFα and IFNγ production. Results are given as percentage of gated MAIT cell population. (A) Representative dot-plots of MAIT cells stimulated with *E. coli* (control) and CPF- or GLP-treated *E. coli* from $n = 6$ donors. (B) Summarized data for the stimulation with CPF- and GLP-treated *E. coli* after normalization to the control (untreated *E. coli*), $n = 6$, mean ± SEM, one-way ANOVA. $^*p < .05$, $^{**}p < .01$ vs. control.

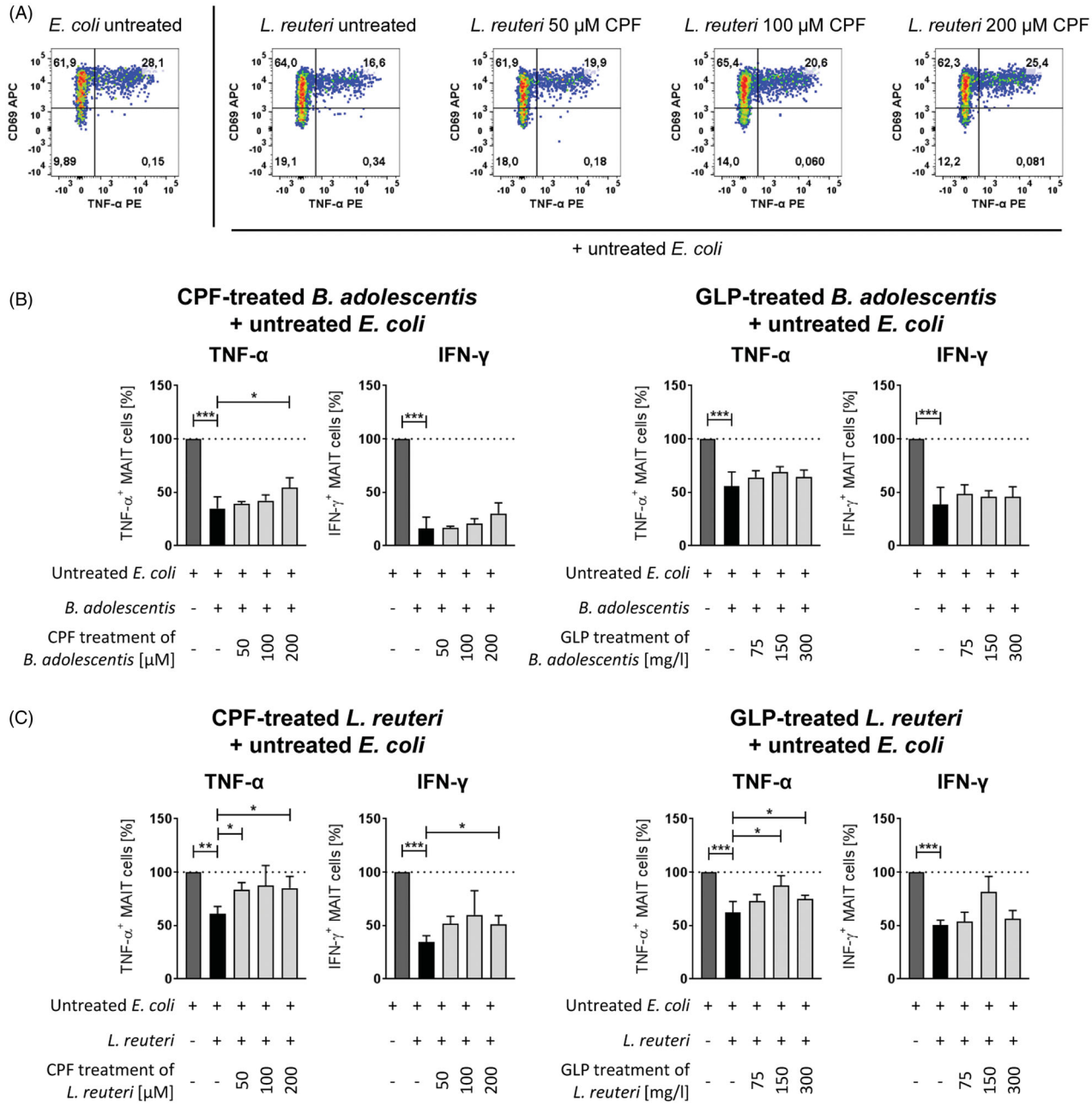

Figure 4. Modulation of *Escherichia coli* (*E. coli*)-induced MAIT cell activation by CPF- or GLP-treated *B. adolescentis* and *L. reuteri*. PBMC were co-stimulated for 6 hr with untreated fixed *E. coli* (30 BpC) and fixed CPF- or GLP-treated (or untreated) (A) *B. adolescentis* (20 BpC) or (B) *L. reuteri* (6 BpC). MAIT cells ($CD3^+CD8a^+CD161^+TCRV\alpha7.2^+$ populations) were detected by flow cytometry. Activation of cells was measured via intracellular staining of TNFα and IFNγ production. Results are given as percentage of gated MAIT cell population. (A) Representative dot-plots of cells (from $n = 6$ donors) stimulated with untreated *E. coli* (control) and in co-stimulation with CPF-treated or untreated *L. reuteri*. (B) Summarized data for co-stimulation with CPF- and GLP-treated *B. adolescentis and L. reuteri* after normalization to control (un-treated *E. coli).* Dark gray bars show response to untreated *E. coli*. Black bars represent untreated co-stimulation control (untreated *B. adolescentis* or *L. reuteri* + untreated *E. coli*), $n = 6$, mean ± SEM, one-way ANOVA. $^*p < .05$, $^{**}p < .01$ vs. control.

In contrast, CPF treatment of *L. reuteri* significantly changed the ability of these bacteria to modulate MAIT cell activation by untreated *E. coli*. Specifically, it was seen that levels of both TNFα- and IFNγ-producing MAIT cells were significantly increased compared to that seen after co-stimulation with the untreated *L. reuteri* (Figures 4(A,C)). Compared with CPF, treatment with GLP led to *L. reuteri* that could induce a slightly higher number of TNFα-producing MAIT cells in the co-stimulation assays than non-GLP treated *L. reuteri* (Figure 4(C)). However, this effect was not observed with regard to IFNγ-producing cells.

Bacterial vitamin production upon CPF and GLP exposure

Concentrations of riboflavin and folate (as end-products of respective biosynthesis pathways) were measured as proxies for the already-known MAIT cell-activating and -inhibiting metabolites. Raw data for untreated as well as CPF- and GLP-treated bacteria are provided in Supplementary Table S4. To better assess the influence of CPF and GLP treatment on folate and riboflavin production, all results were normalized to untreated control values. Regarding ribo-flavin production, a high concentration was seen in *E. coli* whereas the concentrations in the

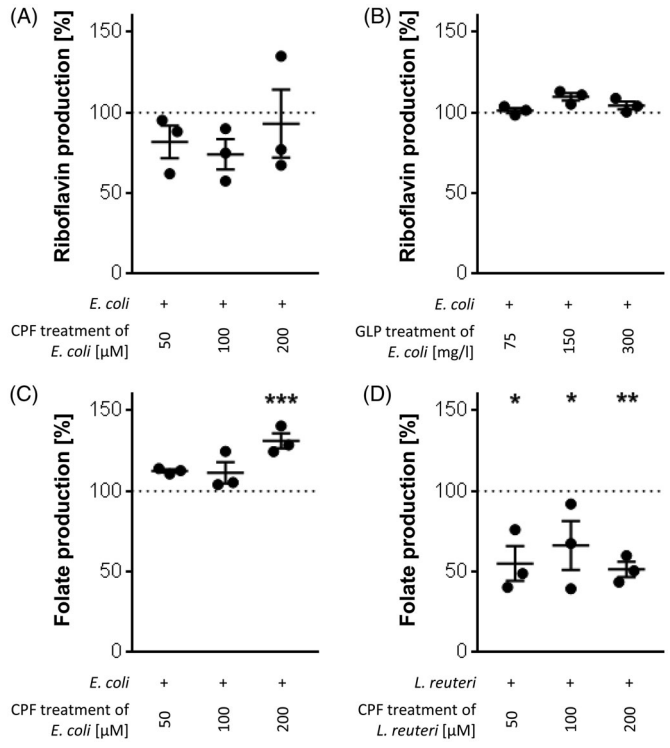

Figure 5. Riboflavin and folate production by CPF- and GLP-treated bacteria. Riboflavin production was measured in *E. coli* lysates after treatment with increasing levels of (A) CPF and (B) GLP. Folate production was analyzed in bacterial lysates of (C) *E. coli* and (D) *L. reuteri* after CPF treatment. Riboflavin and folate production were normalized to the control (bacteria without chemical treatment). $N = 3$, mean ± SEM, one-way ANOVA. $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$ vs. control.

lysates of *B. adolescentis* and *L. reuteri* were rather low. CPF treatment (at 100 µM) of the *E. coli* led to lower riboflavin production (Figure 5(A), Supplementary Table S4); in contrast, after GLP treatment (150 mg/L), production trended higher (Figure 5(B)). With *B. adolescentis* and *L. reuteri*, riboflavin production was not affected by CPF or GLP treatment.

Folate was detected in all bacterial strains, with *E. coli* exhibiting the highest production. CPF treatment led to a significantly higher folate concentration in *E. coli* compared to the control (Figure 5(C)). However, *E. coli* folate production was not affected by GLP (Supplementary Table S4). The CPF or GLP treatment did not alter *B. adolescentis* folate production. With *L. reuteri*, there was nearly 50% lower formation of folate after CPF treatment (compared to the control) whereas GLP treatment imparted no significant effect (Figure 5(D)).

Proteomic analysis of CPF-treated E. coli

The strongest effect of pesticide treatment regarding MAIT cell activation was seen with CPF-treated *E. coli*. Therefore, proteomic analysis was performed after CPF treatment of this strain. LC-MS/MS based shotgun proteomics of CPF-treated *E. coli* revealed 2033 proteins in total, with very similar numbers of proteins per treatment group and sample (Supplementary Figure S4-A). NMDS modeling control based on *E. coli* protein label-free quantification (LFQ) values revealed a trend ($p = .082$, PERMANOVA) in separation of the global proteomes of the three treatment groups and the control (Supplementary Figure S4-C). Pair-wise NMDS modeling showed significant separation between samples treated with the highest CPF concentration (200 µM) and the control ($p = .027$, PERMANOVA), but not at the lower CPF concentrations (Supplementary Figures S3-D, -E, and -F). The LFQ protein values were correlated with the concentration of CPF. In total, 317 *E. coli* proteins significantly correlated ($p < .05$) with CPF concentration; 180 were negatively-correlated to CPF concentration while 137 exhibited positive correlation (Supplementary Figure S4-B). All known *E. coli* proteins of the folate biosynthesis pathway were detected. Two proteins had significant negative correlation to CPF concentration, i.e. *p*-aminobenzoate synthetase component I (PabB, K01665, $r = -0.5942$, $p = .0022$) and dihydropteroate synthase (FolP, K00796, $r = -0.5216$, $p = .0090$) (Figure 6(A)). Furthermore, all *E. coli* proteins known to belong to the riboflavin biosynthesis pathway were identified. Riboflavin synthase (RibE, K00783, $r = -0.5667$, $p = .0038$) was observed to have a negative correlation to CPF concentration (Figure 6(B)).

Discussion

The hypothesis of the present investigation was that pesticides might alter the immuno-modulatory function of commensal bacterial strains. To test this hypothesis, MAIT cells were examined since it is known that activation of these cells is modulated by bacterial metabolites of the B vitamin pathway. Three commensal bacterial strains, *E. coli*, *B. adolescentis* and *L. reuteri*, were selected and the frequently-used pesticides CPF and GLP were chosen for their treatment. MAIT cell activation was assessed via induction of intracellular cytokine production after stimulation of PBMC with treated or untreated bacteria. Concentrations of riboflavin and folate were measured in bacterial lysates after CPF or GLP treatment. Additionally, a proteomic analysis was performed with CPF-treated *E. coli*.

For this study, pesticide concentrations were chosen that were used in recent publications concerning microbiota. Harishankar et al. (2013) showed that CPF at a dose of ≤ 285 µM was tolerated by all tested strains. Therefore, concentrations in a similar range (i.e. 50–200 µM) were used here. Regarding the GLP concentrations, published data are rather diverse, i.e. 5 mg/L–5 g/L (Shehata et al. 2013; Motta et al. 2018; Nielsen et al. 2018). However, sufficient concentra-tions of aromatic amino acids in the bacterial environment can strongly limit the effect of GLP on bacteria (and prevent anti-microbial effects [Nielsen et al. 2018]). Therefore, in the present study, media containing aromatic amino acids (to mitigate anti-microbial effects) and GLP concentrations of 75–300 mg/L were employed.

Certainly, there might be unknown (non-toxicity related) effects of GLP and CPF on bacterial metabolism leading to an alteration of MAIT cell activation. For humans, acceptable daily intakes (ADI) of these compounds are 0.01 mg CPF/kg and 1.0 mg GLP/kg (according to WHO [2004 and 2016, respectively]). In a 70 kg human with 1.2 L gut volume, this corresponds to a 25–100 (CPF)- and 1.25–5.00 (GLP)-fold lower dose compared to the levels employed in the current study. Though the levels used here exceeded these ADI, the underlying aim was to analyze if these pesticides impact the bacterial metabolism at all. Hence, a high-dose and short-term exposure (i.e. 16 hr) paradigm was used. It would be of great interest to investigate long-term low-dose exposures in future experiments, since contaminated foods might be ingested over years.

Here, toxicity assays for both pesticides were first performed to ensure bacterial viability. These assays revealed that the concentrations of CPF (50–200 µM) and GLP (75–300 mg/L) used did not inhibit growth of *E. coli*, *B. adolescentis* and *L. reuteri*.

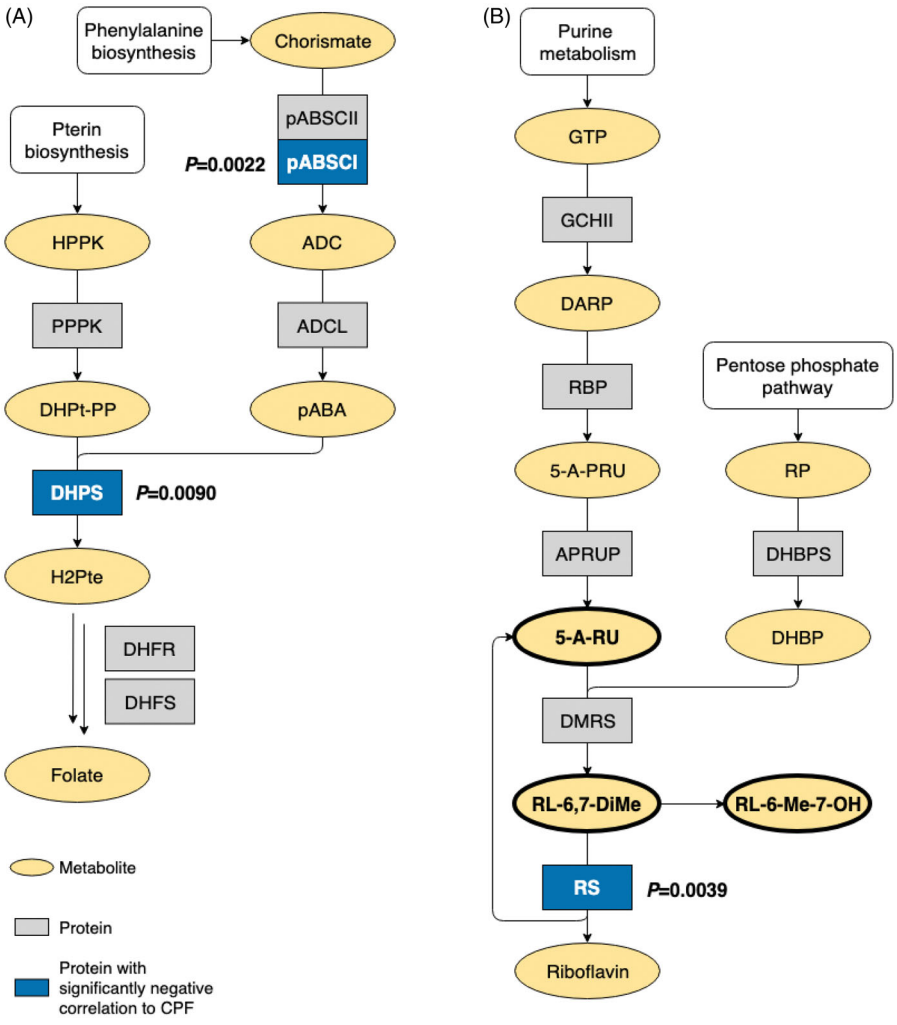

Figure 6. Folate and riboflavin metabolism of CPF-treated *E. coli*. The *E. coli* proteome was analyzed in bacteria that were either untreated (solvent) or CPF-treated (50-200 μM) for 16 hr. Proteins of the (A) folate and (B) riboflavin biosynthesis pathways are represented by rectangles and are either gray (protein detected but not influenced by CPF treatment) or blue (protein exhibits significant negative correlation with CPF, $p < .05$). p-Values are indicated when a significant correlation with CPF treatment was seen. Metabolites are represented by yellow ellipses. MAIT cell activating metabolites in riboflavin pathway are in bold. 5-A-PRU, 5-amino-6-(5-phosphoribosylamino)-uracil; 5-A-RU, 5-amino-6-D-ribitylaminouracil; ADC, 4-amino-4-deoxychoris-mate; ADCL, 4-amino-4-deoxychorismate lyase; APRUP, 5-amino-6-(5-phospho-D-ribityl-amino)-uracil phosphatase; DARP, 2,5-diamino-6-(5-phosphoribosylamino)-4-pyrimi-dinone; DHBP, 3,4-dihydroxy-2-butanone-4-phosphate; DHBPS, 3,4-dihydroxy-2-butanone-4-phosphate synthase; DHFR, dihydrofolate reductase; DHFS, dihydrofolate synthase/folyl-poly-glutamate synthase; DHPS, dihydropteroate synthase; DHPt-PP, 6-hydroxymethyl-7,8-dihydrop-terin diphosphate; H2Pte, 7,8-dihydropteroate; DMRS, 6,7-dimethyl-8-ribityllumazine synthase; GCHII, guanosine-5-triphosphate cyclohydrolase II; GTP, guanosine-5-triphosphate; HPPK, 6-hydroxymethyl-7,8-dihydropterin; pABA, *p*-aminobenzoate; pABSCI, pABA synthetase compo-nent I; pABSCII, pABA synthetase component II; PPPK, 6-hydroxymethyl-7,8-dihydropterin pyrophosphokinase; RBP, riboflavin biosynthesis protein; RL-6,7-DiMe, 6,7-dimethyl-8-D-ribityllumazine; RL-6-Me-7-OH, 7-hydroxy-6-methyl-8-D-ribityllumazine; RP, ribulose-5-phosphate; RS, riboflavin synthase.

These outcomes differ slightly from those in earlier studies that showed that GLP (at some concentrations) could impair the growth of *E. coli* (5000 mg/L) and *B. adolescentis* (75 mg/L), as well as some *Lactobacilli* strains (600 mg/L) (Shehata et al. 2013). Those effects were caused by inhibition of 5-enolpyruvylshiki-mate-3-phosphate synthase, leading to insufficient amounts of aromatic amino acid formation by the bacteria (Motta et al. 2018). As already noted, all bacteria culture media used in the present study contained aromatic amino acids as part of the yeast extract (Supplementary Table S1) (Martini et al. 1979). Therefore, apart from the concentrations here being lower than the "toxic ones" in the Shehata et al. study, one could assume that the findings of a lack of GLP-mediated toxicity here could have been due to sufficient concentrations of aromatic amino acids being present in the culture media. Regarding CPF, it has been shown that *E. coli* can grow even in the presence of high concentrations of CPF (up to 4 mM), tolerate CPF for up to 15 days, and contribute to the degradation of the agent itself (Harishankar et al. 2013). Because of the lack of "lethality" of the test pesticides here, it can be concluded that the observed effects on MAIT cell activation here were not due to reduced bacterial viability but rather likely via secondary effects of the pesticides on bacterial metabolism (which in turn affected formation of known MAIT cell inhibiting and activating metabolites).

The present study findings also revealed that CPF-treated *E. coli* induced a significantly higher number of TNFα- and IFNγ-producing MAIT cells. This enhanced MAIT cell activation might be due to the presence of more activating bacterial metabolites, less inhibiting bacterial metabolites, or a combination of both. To prove this, the *E. coli* proteome was evaluated after CPF treatment.

Exposure to 200 μM CPF caused significant alterations in the global *E. coli* proteome that were not observed at lower concentrations (Supplementary Figure S4-F). In addition, the proteomic analysis of the CPF-treated *E. coli* revealed a significantly negative correlation of CPF treatment with riboflavin synthase (RS,

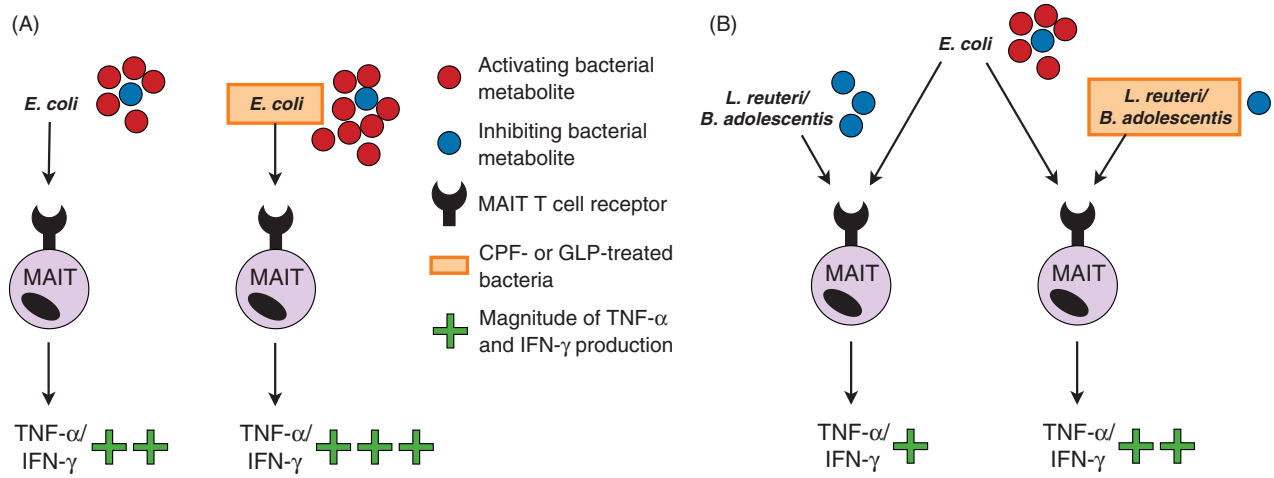

Figure 7. Schematic of MAIT cell stimulation assays with untreated and CPF- or GLP-treated *E. coli*, *B. adolescentis*, and *L. reuteri*. (A) MAIT cells stimulated with untreated *E. coli* or CPF- or GLP-treated *E. coli* (orange) in single stimulation assays. (B) MAIT cells were stimulated with untreated *B. adolescentis* or *L. reuteri* or CPF- or GLP-treated *B. adolescentis* or *L. reuteri* (orange) in single stimulation or co-stimulation assays with untreated *E. coli*. MAIT cells ($CD3^+CD8a^+CD161^+TCRV\alpha7.2^+$ populations) were detected by flow cytometry. Activation measured via intracellular staining of TNFα and IFNγ production. Green crosses show levels of TNFα and IFNγ production. Amount of activating and inhibiting bacterial metabolites is indicated as red and blue circles, respectively.

Figure 6(B)), a key enzyme of the riboflavin biosynthesis pathway. Down-regulation of this enzyme can lead to reduced riboflavin production. In line with this, there was a trend for reduced riboflavin production by CPF-treated *E. coli* in the current study. Since expression of all other enzymes of the riboflavin biosynthesis pathway did not correlate with CPF treatment, the down-regulation of this enzyme could have conceivably led to an accumulation of MAIT cell-activating riboflavin metabolites. As a consequence, CPF-treated *E. coli* may cause stronger MAIT cell activation, leading to an enhanced number of activated cells and as was noted, increases in TNFα and IFNγ production.

In addition to activating riboflavin metabolites, the levels of inhibitory folate metabolites synthesized by *E. coli* might have been altered by CPF. The proteomic analysis showed that expression of a key enzyme of folate biosynthesis, dihydropteroate synthase (DHPS), was significantly negative correlated with CPF treatment. Reduced DHPS levels could lead to lower folate production by the treated *E. coli*. Oddly, significantly stronger folate production by *E. coli* after CPF treatment was observed (Supplementary Table S1). Previous studies have shown that the DHPS is competitively feedback inhibited by its own product dihydropteroate, and by folate and some of its related molecules (Prabhu et al. 1997; Vinnicombe and Derrick 1999). Therefore, the observed negative correlation of this enzyme with CPF treatment in *E. coli* might be due to product inhibition by folate itself, since folate production is higher in CPF-treated *E. coli*.

Despite the increased folate content after CPF treatment, it is difficult to estimate whether levels of MAIT cell-inhibiting folate metabolites were altered. Though speculative, it might be that degradation of folate to MAIT cell-inhibiting metabolites was impaired by CPF, leading to lower amounts of these metabolites – and as a consequence, to an enhanced number of activated MAIT cells. In addition, MAIT TCR exhibits an increased affinity to MR1 presenting activating riboflavin metabolites compared to MR1 with inhibiting folate metabolites (Patel et al. 2013). Thus, one might assume that the observed enhanced MAIT cell activation by CPF-treated *E. coli* was probably mostly driven by an accumulation of the riboflavin metabolites.

In comparison to CPF, GLP treatment had a smaller influence on the *E. coli*-mediated MAIT cell activation but did not affect vitamin production. This led us to assume that this pesticide may not (or only slightly) impact upon *E. coli* metabolism.

Regarding the other bacterial strains, the current study showed that untreated as well as CPF- or GLP-treated *B. adolescentis* and *L. reuteri* did not induce MAIT cell activation *per se*, but inhibited *E. coli* induced activation (in co-stimulation assays). Thus, the main question this generated was whether CPF- and GLP-treatment might change the inhibitory capacity of these strains during co-stimulation with untreated *E. coli*. The findings showed there was a moderate increase in TNFα- but not IFNγ-producing MAIT cells after co-stimulation with GLP-treated *L. reuteri* compared to with untreated *L. reuteri*. CPF-treated *L. reuteri* led to a significant increase of both TNFα- and IFNγ-producing MAIT cells, indicating a reduced MAIT cell inhibiting capacity after treatment. CPF-treated *B. adolescentis* also led to a significant increase of TNFα- and a trend of an increase in IFNγ-producing MAIT cells in co-stimulation assays. Taken together, the findings let us assume that CPF treatment especially might impact bacterial metabolite formation by *B. adolescentis* and *L. reuteri*, leading to alterations in the inhibition of MAIT cell activation (Figure 7).

Concerning riboflavin production by *B. adolescentis* and *L. reuteri*, no changes were observed after CPF or GLP treatment (Supplementary Table S1). Since the concentration of riboflavin in the bacterial lysates of these strains was in general very low, production of folate might be more important for these bacteria. Indeed, analyses of folate content showed that the observed reduction in levels of MAIT cell inhibition by CPF-treated *L. reuteri* was accompanied by significantly lower folate production. This might lead to smaller amounts of inhibitory folate metabolites, depending on any impact of the pesticides on regulation of folate degradation by *L. reuteri*. Regardless of how precisely CPF and GLP treatment of *B. adolescentis* and *L. reuteri* led to a reduction of their normal inhibition of MAIT cell activation, these changes in immuno-regulatory impact of these bacteria resulted in increased numbers of TNFα- and IFNγ-producing MAIT cells. In a live host, this would suggest a directing of immune responses toward more inflammation.

Limitations

The authors are keenly aware that a key limitation of the current study was that the experiments were performed with peripheral blood cells (PBMC) and not with MAIT cells from the gut where pesticides would be presumed to act first in an exposed host. Because gut MAIT cells have differences in activation marker expression (relative to those on PBMC) (Booth et al. 2015), differing reactions of gut MAIT cells to CPF- and GLP-treated bacteria cannot be as yet be ruled out. It is also important to mention that the current data represents results in a simple model (cultured bacteria/cells). Regardless, these outcomes should promote further studies in more complex bacterial communities and also in intact animal models.

Conclusions

The results of the current study indicate that CPF – and to lesser extent GLP – might alter bacterial metabolism, leading to imbalances in levels of activating\inhibiting bacterial metabo-lites that could impact on inflammatory immune responses. Inflammation is associated with a variety of non-communicable diseases including multiple sclerosis, inflammatory bowel disease, obesity and asthma, and alterations in MAIT cell function and distribution are also known to be associated with these diseases. Therefore, the present findings lead us conclude that bacterial treatment with CPF and GLP, resulting in an enhanced inflammatory cytokine production by MAIT cells, might potentially contribute to the development and/or progression of inflammatory immune system-based diseases.

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Disclosure statement

The authors report no conflicts of interest. The authors alone are responsible for the content of this manuscript.

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