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Comparative immunomodulatory effects in mice and in human dendritic cells of five bacterial strains selected for biocontrol of leafy green vegetables

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ABSTRACT

The market for ready-to eat vegetables is increasing, but unfortunately so do the numbers of food-borne illness outbreaks related to these products. A previous study has identified bacterial strains suitable for biocontrol of leafy green vegetables to reduce the exposure to pathogens in these products. As a tentative safety evaluation, five selected strains (*Rhodococcus cerastii* MR5x, *Bacillus coagulans* LMG P-32205, *Bacillus coagulans* LMG P-32206, *Pseudomonas cedrina* LMG P-32207 and *Pseudomonas punonensis* LMG P-32204) were individually compared for immunomodulating effects in mice and in human monocyte-derived dendritic cells (MoDCs). Mice receiving the two *B. coagulans* strains consistently resemble the immunological response of the normal control, and no, or low, cell activation and pro-inflammatory cytokine expression was observed in MoDCs exposed to *B. coagulans* strains. However, different responses were seen in the two models for the Gram-negative *P. cedrina* and the Gram-positive *R. cerastii*. Moreover, *P. punonensis* and *B. coagulans* increased the microbiota diversity in mice as seen by the Shannon-Wiener index. In conclusion, the two strains of *B. coagulans* showed an immunological response that indicate that they lack pathogenic abilities, thus encouraging further safety evaluation and showing great potential to be used as biocontrol agents on leafy green vegetables.

1. Introduction

The consumption of ready-to-eat leafy green vegetables has seen a continuous rise during the latest decades (Campos et al., 2013; Mercanoglu Taban and Halkman, 2011). Despite positive health effects, there is an underlying safety issue with these products that the consumer might not be aware of, and an increasing amount of food borne disease outbreaks from leafy greens has been reported (Little and Gillespie, 2008; Olaimat and Holley, 2012). Uhlig, E, C Olsson (Uhlig et al., 2017) found that ready-to-eat leafy green vegetable products may contain several members with pathogenic potential that are not possible to wash away in a common household setting. Contamination with potential pathogens can occur at every stage of processing, from pre-harvest to the kitchen of a consumer (Mercanoglu Taban and Halkman, 2011), and the hazard potential is high, because there is no processing step that eliminates contaminants. Additionally, damaged leaves act as a strong growth enhancer for pathogens (Koukkidis et al., 2016).

We have previously isolated and identified bacterial species from the native microbiota of leafy green vegetables that have potential to be used as biocontrol agents in order to prevent establishment and growth of pathogenic bacteria on the leaves (Uhlig et al., 2021). This approach aims to reduce the risk of food-borne illness after consumption of these products. These strains are part of the commensal microbiota, which means that humans are normally exposed to them during consumption of these products. However, as biocontrol agents, these strains may be consumed in higher concentrations.

To be able to use the selected bacterial strains for biological control, the present study serves as a first step to evaluate their safety, in respect

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to immunological responses and microbiota changes, in a mouse model and in human monocyte-derived dendritic cells (MoDCs).

It is generally accepted that the bacterial flora in the gut strongly influences the outcome of the immune response, but an obstacle in using rodents for microbiota analysis is the individual variations in the compositions even within a single in-bred strain. As the initial composition of the microbiota is critical for the experimental outcome, a standardized pre-treatment with the aim to equalize the intestinal flora prior to antagonist administration may be used (Linninge et al., 2014).

Dendritic cells (DCs) are antigen presenting cells and have a central role in linking innate and adaptive immunity. Their expression of pattern recognition receptors (PRRs) allows them to recognize potentially dangerous molecules linked to e.g. an infection and in consequence mount an appropriate immune response. DCs can in this context undergo maturation, where they change their expression of cell surface markers and soluble molecules such as cytokines, thus influencing the immune response underway. Several distinct human DC populations exist, and both cell lines and MoDCs are commonly used as *in vitro* models. DC *ex vivo* populations are difficult to obtain and suffer from DC scarcity. Several studies such as Alcántara-Hernández, M, R Leylek (Alc á ntara-Hern á ndez et al., 2017) and Lundberg, K, A-S Albrekt (Lundberg et al., 2013) describe the complexity and different functionality of DC models as well as the heterogeneity in primary cells due to donor-donor variation (Lundberg et al., 2013; Kelly et al., 2017).

2. Materials and methods

2.1. Mouse model

2.1.1. Animals

Seventy wild type female C57BL/6N mice (Charles River Laboratories, Germany) were kept under standardized conditions in the animal facility and acclimatized for 7 days before start of the experimental protocol. The consumption of water and feed was provided ad libitum. The water consumption was measured every day throughout the study, and the feed consumption was registered at the end of the study. Body weights were registered at start, after pre-treatment and at the end. The animals were divided into seven groups (10 animals each, 5 animals per cage), normal control (NC), treatment control (TC) and five treatment groups (Treatment group *Pseudomonas* 1, (TP1), Treatment group *Pseudomonas* 2 (TP2), Treatment group *Rhodococcus* (TR), Treatment group *Bacillus* 1 (TB1) and Treatment group *Bacillus* 1 (TB2)), see Fig. 1 for the experimental design. Groups TC, TP1, TP2, TR, TB1 and TB2 received a pre-treatment with antibiotics and the non-pathogenic *E. coli* strain CCUG29300T to equalize the microbiota (Linninge et al., 2014). Antibiotics and 2% (v/w) fructose was added to the drinking water for the first three days. An average dose of 5.2 mg metronidazole (Sanofi AB, Stockholm, Sweden), 3.5 mg amoxicillin (Mylan AB, Stockholm, Sweden) and 2.1 mg clindamycin (Orifarm Generics A/S, Odense, Denmark) was consumed by each mouse. Thereafter, *E. coli* CCUG29300^T (10^8 CFU/ml, diluted in Hogness' freezing medium (Ahrn é et al., 1989) was added to the drinking water for 2 days. Group N received only fructose the first three days, and only freezing medium the next two days.

After the pre-treatment, the treatment groups received one bacterial strain each (TP1: *Pseudomonas cedrina* LMG P-32207, TP2: *Pseudomonas punonensis* LMG P-32204, TR: *Rhodococcus cerastii* MR5x, TB1: *Bacillus coagulans* LMG P-32205, TB2: *Bacillus coagulans* LMG P-32206) and freezing medium in the water (10^8 CFU/ml), group TC and NC received only freezing medium for 16 days. All drinking water preparations were freshly made each day.

On day 21, the animals were put under anesthesia with 1.0 mg/kg bodyweight medetomidine (Dormitor® Vet, Orion Pharma Animal Health, Espoo, Finland) and 75 mg/kg bodyweight ketamine (Ketalar, Werner Lambert Nordic AB, Solna, Sweden) by intra-peritoneal injection. Under aseptic technique, a laparotomy was performed through a midline incision. Arterial blood was collected, allowed to clot for 2 h, centrifuged (3000 rpm, 3 min, 4°C), and the serum was frozen at −80°C for later analysis of cytokines/chemokines. Mesenteric lymph nodes (MLNs) and Peyers' patches (PPs) were isolated and transferred to tubes containing Hank's balanced salt solution (HBSS) (Biowest, Nuaillé, France) and kept on ice until analysis by flow cytometry. The spleen and

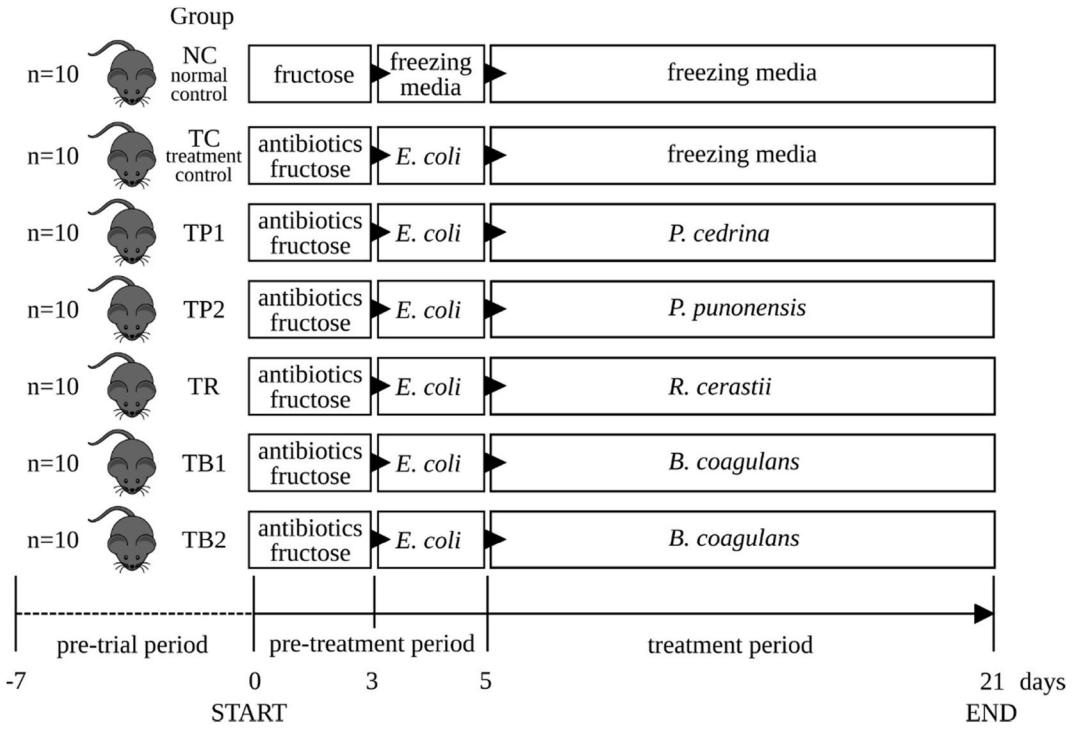

Fig. 1. Experimental design of the animal study. Water consumption was measured daily, and feed consumption at the end. Body weights were registered at start, after pre-treatment and at the end.

the luminal content of small intestine and colon were carefully collected and weighed, and the small intestine and colon tissue was rinsed with isotonic saline. All samples were transferred to sterile tubes for myeloperoxidase and microbiota analysis. The animals were then euthanized by pentobarbital injection (2 ml/kg). The blood and intestine samples were frozen at −81 °C. The MLNs and PPs were kept on ice and immediately analyzed by flow cytometry. The experimental design was approved by the Malmö-Lund regional Animal Ethics Committee (reference number 5.8.18–06202/2017) and the experiments adhered to the national guidelines for the care and use of animals (Swedish Animal Welfare Act, 1988:534).

2.1.2. Myeloperoxidase (MPO)

MPO, an enzyme found in neutrophils and a marker for local intestinal inflammation, was measured in small and large intestinal mucosa with a protocol based on the method from Schultz, J, K Kaminker (Schultz and Kaminker, 1962). Samples were frozen in liquid nitrogen and weighed prior to homogenization in 1 ml potassium phosphate buffer (20 mM, pH 7.4) for 60 s. The homogenate was thereafter centrifuged (14000 rpm, 10 min) and the pellet was re-suspended in 50 mM PBS (pH 6.0) with 0.5% hexadecyltrimethyl-ammonium bromide. The sample was freeze-thawed and then sonicated for 90 s and kept in water bath at 60 °C for 2 h. After centrifugation (14000 rpm, 10 min) the 45 μl of supernatant was transferred to a 96-well plate, 150 μl TMB (3,3′, 5,5′ tetramethylbenzidine) substrate (BD Opt EIA™, BD Biosciences, San Diego, CA, USA) added to each well and incubated in the dark for 15 min. The reaction was then terminated by addition of 100 μl 0.5 M H_2SO_4 per well and the samples were analyzed spectrophotometrically at 450 nm. MPO (Sigma-Aldrich, St. Louis, MO, USA) was used as standard and values expressed as units MPO/g tissue.

2.1.3. Terminal restriction fragment length polymorphism (T-FRLP)

Microbial community structure was determined by T-RFLP. DNA was extracted from large and small intestinal content by adding 500 μl PBS buffer to 50 mg sample and incubated at room temperature for 10 min. Thereafter, a bead beating step was performed for 45 min at 4 °C on an Eppendorf mixer (Model 5432, Eppendorf, Hamburg, Germany) following a centrifuge step (30 s, 3000 rpm). Then 200 μl of the supernatant was transferred to a sterile sample tube in the EZ1 DNA tissue kit (Qiagen, Sollentuna, Sweden), and DNA was extracted according to manufacturers' instructions on a Biorobot EZ1 workstation (Qiagen). PCR and restriction endonuclease (MSPI) digestion was performed as previously described (Sand et al., 2015). The digested amplicons were analyzed on an ABI 3130 xl Genetic analyzer (Applied Biosystems, Foster City, CA, USA) with internal size standard GeneScan LIZ 600 (range 20–600 bases, Applied Biosystems) at DNA lab (SUS, Malmö, Sweden). Data was analyzed with GeneMapper software version 4.0 (Applied Biosystems) with local southern algorithm. Terminal restriction fragments (T-RFs) were resolved between 40 and 580 bases considering that four internal standards were required for accurate sizing of an unknown T-RF. The relative area percentage was calculated for each T-RF which was used for diversity index calculation.

2.1.4. Multiplex cytokine/chemokine analysis

Quantitative measurements of cytokines and chemokines (IFN-γ, IL1-β, IL-2, IL-4, IL-5, IL-6, KC/GRO, IL10, IL12p70, TNF-α), were performed in serum. Frozen samples were thawed and added in duplicates in 1:2 dilutions to 96-well V-plex Proinflammatory Panel 1 (mouse) plates (Meso Scale Diagnostics, LLC, Rockville, MD, USA) according to manufacturer's instructions. The plates were read using MSD Sector S 600 plate reader (Meso Scale Diagnostics, LLC).

2.1.5. Flow cytometry

Peyers' patches and mesenteric lymph nodes were transferred to HBSS, cut into 1 mm pieces, centrifuged and supernatant and fat were removed. The tissue was then digested with Collagenase P (0.8 mg/ml, Sigma-Aldrich, St. Louis, MO, USA), Dispase II (3.2 mg/ml, Sigma-Aldrich) and DNAse I (0,1 mg/ml Sigma-Aldrich) for 10 min at 37 °C. The supernatant was collected, and digestion was repeated twice until all tissue was digested. After centrifugation (1400 rpm, 5 min, 4 °C), the supernatant was removed, resuspended in HBSS (Biowest, Nuaillé, France) with 10% fetal bovine serum (VWR, Radnor, USA) and filtered (40 μm cell strainer (VWR)) The flow-through was centrifuged (1400 rpm, 5 min, 4 °C), supernatant removed and the pellet resuspended in HBSS. A fraction of the cells was stained with Turks' (Merck KGaA, Darmstadt, Germany) and counted. The suspension was diluted to 10^6 cells in HBSS and stained with the following antibody (ab) combinations overnight at 4 °C: TLR2/CD11c/F4:80/TLR4 (panel 1); CCR9/CD4/CD69/CD8a (panel 2); CD4/CD69/CD25/FoxP3 (panel 3) (all ab come from eBioscience, Inc., San Diego, CA, USA). All panels contained CD16/CD32 ab to block non-antigen-specific Fc binding. For intranuclear FoxP3 (eBioscience, Inc., San Diego, CA, USA) staining, cells were fixed and permeabilized according to manufacturer's instructions and then resuspended in FACS buffer (eBiosciences).

Unstained cells were used as control, and for compensation, VersaComp Antibody Capture Bead kit (Beckman Coulter Inc., Brea, CA, USA) was used. The cells were washed once with FACS buffer and run on the Cytoflex flow cytometer (Becton Dickinson, Mountain view, CA, USA). Data analysis was performed with Cytexpert 2.0 (Becton Dickinson) software with assessment of 50 000 events per sample. Lymphocyte populations from both PPs and MLNs were first gated based on forward (FSC) and side scatter (SSC) properties. From this gate, singlets were chosen based on FSC and FSC-Width. For panel 1, macrophages and DCs were identified from the singlets by positive staining for F4/80 and CD11c respectively, those gates were then further used to analyze populations expressing TLR2 and/or TLR4. For panel 2, $CD8^+$ and $CD4^+$ cells were selected from the singlets, and individually gated for gut homing and activation, CCR9 and CD69 expression. For panel 3, activated and resting regulatory T cells were selected from the singlets by Foxp3+$CD69^-$and Foxp3+$CD69^+$. The gating strategy is visualized in Supplementary Figure S1.

2.2. In vitro study using human monocyte-derived dendritic cells

2.2.1. Bacterial solutions

The same test strains as used in the animal study (section 2.1.1) were prepared in PBS 10^9 CFU/ml and heat-inactivated at 70 °C for 30 min. Heat-inactivation was confirmed by a viability control on agar for 5 days and no colony growth was detected. In addition, three different strains of *E. coli*; *E. coli* CCUG29300^T, and two strains isolated from Romaine lettuce and identified to closest type strain *Escherichia/Shigella flexneri*, were prepared in the same way as the other bacterial solutions and were used for comparison.

2.2.2. Isolation and differentiation of monocytes to MoDC

Human peripheral blood mononuclear cells were isolated from leucocyte concentrate (Lund University Hospital, under permission 2017:02) using gradient centrifugation (Ficoll-Paque ™ (Cytiva, Uppsala, Sweden), peripheral blood to Ficoll ratio: 1:1). $CD14^+$ cells were isolated using anti-human CD14 magnetic microbeads (Miltenyi Biotec B.V. & Co. KG, Bergisch Gladbach, Germany) and the results confirmed by flow cytometry. Cells were then grown in R10 medium (RPMI 1640 (HyClone™, Cytiva), 10% FCS (Life Technologies, Carlsbad, CA, USA) 2 mM L-glutamine (Cytiva), 50 μg/ml Gentamicin (Sigma-Aldrich, St. Louis, MO, USA) in six-well plates. Cytokines human recombinant GM-CSF (PeproTech, Hamburg, Germany) (150 ng/ml) and IL-4 (50 ng/ml) (PeproTech) were added to the medium in order for monocytes to differentiate into MoDCs. Cells were cultured at 37 °C and in the presence of 5% CO_2 for 8 days; on day 3 and 6 half the medium was changed.

2.2.3. Stimulation of MoDCs with bacterial preparations

Differentiation into MoDCs was confirmed on day 7 of culture, by staining with CD14 (Dako, Santa Clara, CA, USA) and CD1a (Dako) ($CD14^{-}CD1a+$ are differentiated). On day 8 of differentiation, MoDCs were counted and bacterial preparations added. The ratio between bacterial cells and MoDCs were 1:1 for flow cytometry analysis shown here; 10:1 and 1:10 were also tested and resulted in similar trends (data not shown). A 10:1 ratio between bacterial cells and MoDCs were chosen for the multiplex cytokine assay as Karlsson, H, P Larsson (Karlsson et al., 2004) have seen an optimal cytokine response at higher bacteria: cell ratio (in their case 50:1). The concentration of cells was 500 000 cells/ml and the cells were seeded in a 24-well plate, 0.5 ml per well. LPS (5 ng/ml, Sigma-Aldrich) was used as positive control, and PBS was used as negative control. Cells were then cultured for 24 h at 37 °C in the presence of 5% CO_2 with R10 medium.

2.2.4. Flow cytometry

After 24 h of stimulation, cells were blocked with mouse IgG (100 μg/ml) (Jackson ImmunoResearch, Ely, UK), washed with MACS buffer (PBS, 2 mM EDTA, 0.5% Bovine Serum Albumin (BSA, Saveen Werner, Malmö, Sweden)) and incubated for 15 min at 4°C with the following antibodies: CD14, CD1a (Dako), HLA-DR, CD86, CD80, CD54 (BD Biosciences), TLR2 and TLR4 (Biolegend, San Diego, CS, USA). Cells were analyzed with BD FACSCanto™ II (BD Biosciences) and 10000 events were collected. Data was analyzed with FCS express 4 (De Novo Software, Glendale, CA, USA); viable cells were gated as shown for an untreated sample in Supplementary Figure S2. Forward and side scatter is used to exclude cellular debris (Gate 1). From gate 1, PE and FITC positive cells were selected. In a second plot, gate 1 was used to select APC and FITC positive cells. The degree of activation of cells treated with bacteria was calculated for each marker according to:

$$X = \frac{\%\text{ofpositivecells} \in \text{sample}}{\%\text{ofpositivecells} \in \text{untreatedcontrol}}$$

For HLA-DR, the percentage of positive cells is near 98% in all samples, so instead the following equation was used, indicating the intensity of the emitted fluorochrome light:

$$X = \frac{\text{medianvalueofsample}}{\text{medianvalueofuntreatedcontrol}}$$

2.2.5. Multiplex cytokine assay (in vitro samples)

Supernatant from cell cultures were collected after 24 h of bacterial stimulation and had a ratio of 10:1 bacterial to human cells. Cells were analyzed for the concentration of ten different cytokines (IFN-γ, IL1-β, IL-2, IL-5, IL-6, IL-8, KC/GRO, IL-10, IL-12p70, TNF-α) with a multiplex immunoassay (Human Custom ProcartaPlex™ 10-plex, Thermo Fisher scientific, Vienna, Austria) according to manufacturer's instructions (exception; beads were vortexed 60 s instead of 10 s). Concentrations of cytokines were then measured in technical duplicates using a Bio-Plex™200 system (Bio-Rad, Hercules, CA, USA).

2.3. Statistical analysis

The statistical calculations of feed and water intake, MPO activity, cytokine/chemokine data, diversity indices and flow cytometry data were performed with SigmaPlot v 13.0 software (SPSS Inc., Chicago, USA). The differences between all groups were assessed by Kruskal–Wallis one-way ANOVA on ranks and the differences between two experimental groups were assessed by a Mann–Whitney rank sum test. Results were considered statistically significant when $p \leq 0.05$. Values are presented as median with 25th and 75th percentiles. The statistical calculations of frequency of occurrence of T-RFs within groups were performed with Fisher's test. The flow cytometry data from the cell culture was evaluated with Friedman's test using GraphPad Prism 8.01 (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. In vivo study in mice

3.1.1. Animals

Animal weights, and feed and water intakes are shown in Table 1. Over the study period of three weeks, animals gained between 1.0 and 1.9 g in weight, and consumed between 61.0 and 63.9 g of feed per animal. The water intake was between 3.6 and 4.0 ml per day and animal. No significant changes in the median values between the groups compared to the treatment control group (TC) could be found for body weight, feed or water intake from start to end, and also not during the treatment period. During the pre-treatment period, an increase in body weight compared to the normal control group (NC) was observed for all groups, except for group TR.

No differences in relative spleen weights were found between the groups [NC; 0.0044 g/g (0.0042–0.0044), K; 0.0041 g/g (0.0040–0.0041) TP1; 0.0042 g/g (0.0037–0.0042), TP2; 0.0042 g/g (0.0039–0.0042) TR; 0.0043 g/g (0.0039–0.0043), TB1; 0.0046 g/g (0.0041–0.0046), TB2; 0.0043 g/g (0.0040–0.0043)].

3.1.2. Myeloperoxidase (MPO)

The levels of MPO, an enzyme found in neutrophils and a marker for local intestinal inflammation, were measured in the small and large intestine. No statistically significant differences in the median values of MPO concentration between the groups could be found in the large or in the small intestine (Supplementary Table S1).

3.1.3. Intestinal microbiota structure by T-RFLP

In the small intestine, 41 terminal restriction fragments (T-RFs) were detected. Seventeen T-RF patterns were significantly different between the treatment groups and the treatment control (Table 2). Two to four T-RFs were only detected in the treatment groups and not in the treatment control. Most new T-RF peaks were detected in group TP1 that received *P. cedrina*, and fewest unique T-RFs were detected in group TP2 for animals receiving P. punonensis LMG P-32204.

In the large intestine, 84 T-RFs were detected. Twenty-five T-RF patterns differed significantly between the treatment groups and the control Table 3. Three to six T-RFs were only detected in the treatment groups and not in the control group. Also here, most new T-RF peaks were detected in group TP1, and fewest unique T-RFs were detected in group TP2.

The microbial diversity was calculated based on the T-RFLP patterns, using the peak area of each sample in proportion to the total area of a sample and can be seen in Table 4. In the small intestine, group TP2 (*P. punonensis*) had higher Shannon-Wiener diversity index than the treatment control group (TC). In the large intestine, group TB2 (*B. coagulans*) had higher diversity index than the treatment control group (TC).

3.1.4. Multiplex cytokine/chemokine analysis

The levels of cytokines/chemokines in mouse serum were measured at the end of the study (Supplementary Table S2). Administration of *P. cedrina* (TP1), and *B. coagulans* (TB1, TB2) significantly elevated levels of IFN-γ compared to treatment control (TC). Additionally, significantly higher levels of KC/GRO were observed for animals receiving *R. cerastii* (TR), and *B. coagulans* strains (TB1, TB2) compared to TC.

3.1.5. Flow cytometry

Mononuclear immune cells from Peyer's patches (PPs) and mesenteric lymph nodes (MLN) were analyzed by flow cytometry. In Fig. 2A, the percentage of activated $CD4^{+}$ immune cells (T cells, DCs, macrophages) in MLN were lower in treatment control (TC) compared to normal control (NC). For animals receiving *P. cedrina* (TP1), populations were even smaller, while for *B. coagulans* groups (TB1, TB2), populations

Table 1
Body weights and feed and water intake. Data is analyzed over the whole experiment period, and also divided into two parts; the pre-treatment period when animals received antibiotics and *E. coli*, and the treatment period when the animals received test strains in the drinking water. Statistical differences between groups were compared with Kruskal-Wallis one-way ANOVA on ranks, and differences between two experimental groups were assessed by Mann-Whitney rank sum test.

Group	NC (normal control)	TC (treatment control)	TP1 *P. cedrina* LMG P-32207	TP2 *P. punonensis* LMG P-32204	TR *R. cerastii* MR5x	TB1 *B. coagulans* LMG P-32205	TB2 *B. coagulans* LMG P-32206
Start to end							
Body weight change (g)[a]	1.6 (0.8–2.2)	1.5 (1.1–1.8)	1.0 (0.2–1.9)	1.9 (0.9–2.1)	1.3 (0.7–2.0)	1.8 (0.8–2.6)	1.9 (0.9–3.2)
Feed intake (g)[b]	62.7 (0.41)	63.5 (1.7)	61.0 (5.4)	62.7 (1.6)	63.2 (0.7)	62.8 (3.1)	63.9 (2.1)
Water intake/ day (ml)[b]	20.0 (19.0–20.0)	20.0 (18.0–22.0)	18.5 (16.0–20.5)	20.0 (18.0–22.0)	20.0 (16.5–23.5)	18.5 (16.0-24-0)	20.0 (18.0–24.0)
Pre-treatment period							
Body weight change (g)[a]	0.2 (−0.5-0.2)	0.6 (0.3–0.9)††	0.5 (0.1–1.2)†	1.0 (0.8–1.7)†††	0.0 (−1.7-1.6)	1.6 (1.3–2.0)†††	1.3 (1.0–1.5)†††
Water intake/ day (ml)[a]	20.0 (20.0–25.0)	8.0 (6.0–34.5)	6.0 (4.0–32.0)	8.0 (7.0–32.0)	10.0 (5.0–31.0)	9.0 (8.0–33.0)	13.0 (3.5–34.5)
Treatment period							
Body weight change (g)[a]	1.3 (0.9–2.5)	0.9 (0.4–1.3)	0.2 (−0.4-1.0)	0.5 (0.0–0.9)	1.3 (0.1–2.3)	0.15 (−0.6-1.1)	0.6 (0.0–1.5)
Water intake/ day (ml)[a]	20.0 (18.5–20.0)	20.0 (18.0-22-0)	19.0 (17.0–20.0)	20.0 (18.0–22.0)	20.0 (17.3–23.0)	19.0 (16.0–23.0)	20.0 (18.3–24.0)

† $P < 0.05$, †† $P < 0.01$ and ††† $P < 0.001$ compared to normal control (NC) in the same row.
[a] Data is expressed as median values of 10 individuals with interquartile range (25–75%).
[b] Data is expressed as average value of two cages (5 animals in each) with standard deviation within paranthesis.

Table 2
Size of T-RFs detected in small intestine that have significantly different frequency of occurrence between the different groups and control (TC). NC: normal control, TC: treatment control, TP1: *P. cedrina* LMG P-32207, TP2: *P. punonensis* LMG P-32204, TR: *R. cerastii* MR5x, TB1: *B. coagulans* LMG P-32205, TB2: *B. coagulans* LMG P-32206. Twenty-four T-RFs were not significant between treatment groups and TC, and are not included in the table. Statistical calculations were performed using Fisher's test.

T-RFs	NC	TC	TP1	TP2	TR	TB1	TB2
82.62	7**	0	1	0	3	3	5
83.55	1	0	9***	3	6*	1	5
84.83	8	8	8	0***	6	9	6
87.26	0*	6	7	0*	0*	0*	0*
90.43	6*	0	1	1	4	1	1
94.38	2	0	6*	0	1	2	1
150.93	1	0	3	0	4	6*	6*
160.51	6*	0	0	1	0	0	0
179.24	0	0	0	6*	0	0	0
184.85	7	10	9	0***	9	9	9
265.90	0	0	1	8***	0	0	2
266.98	9	10	5	0***	10	10	10
284.12	1	0	5*	0	7**	8***	8***
290.28	5*	0	0	0	0	0	0
491.45	0	0	1	0	6*	1	0
495.44	0*	6	8	0*	9	0*	0*
574.30	3	0	6*	0	7**	6*	6*

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared to treatment control, TC.

Table 3
Size of T-RFs detected in large intestine that have significantly different frequency of occurrence between the different groups and control (TC). NC: normal control, TC: treatment control, TP1: *P. cedrina* LMG P-32207, TP2: *P. punonensis* LMG P-32204, TR: *R. cerastii* MR5x, TB1: *B. coagulans* LMG P-32205, TB2: *B.* LMG P-32206. Fifty-nine T-RFs were not significant between treatment groups and TC, and are not included in the table. Statistical calculations were performed using Fisher's test.

T-RFs	NC	TC	TP1	TP2	TR	TB1	TB2
82,62	0***	9	0***	5	0***	0***	0***
83,55	0	0	8***	1	3	2	5
84,83	10	10	9	6	0***	9	9
87,26	10	10	7	0***	0***	0***	9
88,23	0	0	8***	4	9***	4	4
90,43	8	8	9	6	6	0***	8
92,39	7*	0	7**	3	1	6*	5
94,38	10	10	9	0***	0***	8	9
131,42	0**	7	0**	0**	0**	0**	0**
141,18	0	0	2	5*	8***	0	1
176,98	4	0	0	2	3	6*	3
207,52	0**	7	6	0**	0**	0**	9
216,84	0*	6	0*	0*	0*	0*	0*
218,54	4	0	4	2	5	6*	6*
220,67	3	0	7**	3	5	6*	6*
276,30	2	0	0	3	7**	3	5
283,16	2	0	7**	5	8***	5	6*
284,12	6*	0	4	5*	4	4	5
285,11	8***	0	2	2	3	1	7**
290,28	0*	6	0*	0*	0*	0*	0*
294,21	0*	6	9	0*	8	6	9
296,05	0*	6	8	0*	8	6	0*
495,44	0	0	10***	5*	8***	6*	7**
543,81	0***	8	0***	0***	0***	0***	8
546,49	10	10	9	4	0***	8	9

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared to treatment control, TC.

were larger than TC, similar to NC.

In Fig. 2B, the activated $CD8^+$ immune cells (cytotoxic T cells) in MLN follow the same pattern as in Fig. 2A.

Fig. 2C shows Gram-positive bacteria induced upregulation (TLR2+) of DCs in PPs. Populations are larger in TC compared to NC, and even larger for the *R. cerastii* group (TR). For the *P. cedrina* (TP1) group, populations are on the same level as for NC. In Fig. 2D showing Gram-negative bacteria induced upregulation (TLR4+) of DCs in PPs, the treatment group populations are of the same size as TC.

In Fig. 2E, TLR2+ macrophages in PPs show larger populations in TC compared to NC, and all treatment groups have smaller populations, similar to NC. Fig. 2F shows TLR4+ macrophages in PPs. TC populations are larger than NC, while for all treatment groups except for TP1, the levels are in the same size range as in NC.

Activated MLN regulatory T cell populations ($CD69^+CD4^+CD25+FOXP3+$) in Fig. 2G was lower in TC compared to NC, and all treatment groups had larger population sizes, similar to NC. The complete set of flow cytometry results can be found in Supplementary Table S3-S5.

3.2. Human monocyte-derived dendritic cells

Before bacterial stimulation on a 24-well plate, MoDCs were found to have a similar, round shape in all wells, with only few single cells attached to the wells. After 24 h of incubation with *P. punonensis* (TP2)

Table 4
Shannon-Wiener (H′) diversity index in the small intestine (SI) and in the large intestine (LI). Data is expressed as median values of 10 individuals with interquartile range (25–75%). NC: normal control, TC: treatment control, TP1: *P. cedrina* LMG P-32207, TP2: *P. punonensis* LMG P-32204, TR: *R. cerastii* MR5x, TB1: *B. coagulans* LMG P-32205, TB2: *B. coagulans* LMG P-32206. Statistical differences between groups were compared with Kruskal-Wallis one-way ANOVA on ranks, and differences between two experimental groups were assessed by Mann-Whitney rank sum test.

Group	Shannon-Wiener (H′), SI[a]	Shannon-Wiener (H′), LI[a]
NC	1.63 (1.53–1.80)**	2.54 (1.99–2.75)
TC	1.22 (0.77–1.37)	2.05 (1.76–2.47)
TP1	1.49 (1.10–1.53)†	2.20 (2.01–2.59)
TP2	1.43 (1.33–1.77)*	2.27 (1.52–2.44)
TR	1.31 (1.08–1.56)†	2.50 (1.94–2.81)
TB1	1.29 (0.99–1.45)†	2.15 (1.85–2.46)
TB2	1.36 (1.25–1.57)†	2.73 (2.51–2.86)**

†$P<0.05$ compared to normal control (NC) in the same column * $p < 0.05$, **$p < 0.01$ compared to treatment control (TC) in the same column.

and *R. cerastii* (TR), MoDCs formed small clusters and were attached to the plate surface, possibly indicating activation. MoDCs incubated with bacteria *P. cedrina* (TP1), and *B. coagulans* (TB1, TB2) had few clusters but also attached to the plate surface. Untreated cells and cells treated with PBS did not form any clusters, but few cells attached to the surface. Cell viability was >95% in all samples.

3.2.1. MoDC activation marker expression assessed by flow cytometry

Human MoDCs derived from five individuals were stimulated for 24 h with controls and bacterial preparations. Dendritic cell activation markers CD80/CD86 and HLA-DR were investigated by flow cytometry and the results are shown Fig. 3. Cells stimulated with *E. coli* 1, 2, 3, *P. punonensis* (TP2) and *R. cerastii* (TR) seemed to cause a higher activation of MoDCs compared to the untreated cells and the cells treated with PBS. A similar pattern is seen for all markers. Most consistent and prominent activation was induced by the different *E. coli* strains (significant compared to control for CD86 Fig. 3A and CD80 expression (Fig. 3B)). *P. punonensis* (TP2) and *R. cerastii* (TR) also led to an increased expression of CD80 (Fig. 3B), which is mirrored by the pattern of CD86 expression (Fig. 3A) and the increase in median fluorescence intensity of HLA-DR expression (Fig. 3C). In contrast, cells exposed to *P. cedrina* (TP1) and *B. coagulans* strains (TB1 and TB2), did not seem to induce a change in the DC activation markers observed here.

3.2.2. Multiplex cytokine profiling

The cell culture supernatant from three donors after bacterial stimulation with a ratio 10:1 bacterial cells to MoDCs was collected to quantify expressed cytokines. In Fig. 4 the concentrations of IL-6 (Fig. 4A), IL-12p70 (Fig. 4B), IL-1β (Fig. 4C) and IFN-γ (Fig. 4D) are presented, remaining cytokine data not shown due to high variation and no clear trends. No statistical evaluation was performed due to low number of replicates.

A similar pattern as observed for MoDC activation emerged: the *E. coli* strains and *P. punonensis* (TP2) and R. cerastii (TR) seemed to be able to stimulate production of IL-6, IL-12p70, IL-1β and IFN-γ compared to neg. control (PBS) (Fig. 4A–D). IL-6 (Fig. 4A) levels also appeared to increase in response to LPS, *P. punonensis* (TP2), *R. cerastii* (TR) and *B. coagulans* (TB1 and TB2) stimulation.

4. Discussion

In a previous study, we found specific bacterial strains suitable for bio control of leafy green vegetables. Those strains have been identified as species that are regarded as non-pathogens to humans and animals. As the first line of safety evaluation, the immunological response in a mouse model and in a human DC model has been investigated. Additionally, a microbiota change assessment was performed in the *in vivo* trial.

In the *in vivo* trial, C57BL/6 mice were used, a strain initially developed and commonly used for immune response analysis (Song and Hwang, 2017; H å kansson et al., 2021; Burri et al., 2021). Female animals were used to obtain comparable results with as few animals as possible, taking into account the three R's in experimental animal research. During the pre-treatment period, all groups displayed a higher weight gain compared to normal control, most likely an effect of a larger water intake at the time of measurement, when the antibiotic administration was over. During the treatment period when the animals received the test strains, the body weight gain was not significantly different between the groups.

Myeloperoxidase (MPO) is an enzyme found in neutrophils and high levels in tissue are correlated with local inflammation (Kristj á nsson et al., 2004). Probiotic strains have previously been noted to lower MPO levels (Chen et al., 2009; Osman et al., 2006). In this study, the test strains did not alter the concentration of MPO in the small or large intestine (Supplementary Table S1). However, a decreasing trend can be observed for groups receiving the Gram-positive *R. cerastii* and *B. coagulans*, possibly indicating a lower grade of neutrophil accumulation in those groups.

Evaluations of immune functions were further approached through flow cytometry of lymphocyte subpopulations, macrophages and dendritic cells in Peyer's patches and mesenteric lymph nodes, as well as through multiplex cytokine profiling of serum samples. When measuring the pro-inflammatory cytokine concentrations in serum, it could be shown that administration of test strains resulted in significantly elevated levels of IFN-γ in groups receiving *P. cedrina* and *B. coagulans* (TP1, TB1 and TB2) compared to treatment control, TC (Supplementary Table S2). Koizumi, S-i, D Wakita (Koizumi et al., 2008) noted that *Lactiplantibacillus pentosus* strongly induces the type-1 (Th1) immune response including IFN-γ, which is critical in its role to fight disease and suppress the type-2 (Th2) immune response related diseases such as allergy. The cytokine KC/GRO is a neutrophil chemoattractant and found to increase in group TR, TB1 and TB2. A previously performed study by Håkansson, Å, N Tormo-Badia (H å kansson et al., 2015) has seen a drastic increase in KC/GRO levels in mice with inflammation induced by dextran sulfate sodium (DSS) compared to healthy control, and according to two studies, probiotic strains seem to decrease KC/GRO levels (Liu et al., 2010; Zhang et al., 2006). However, secretion of the human homologue of KC, IL-8, has recently been seen to also increase in response to commensal *E. coli* (Saxena et al., 2018), which was administered as pretreatment in this study. It has also been observed that probiotic strains are capable of activating neutrophils as part of an anti-tumor response and a polarization towards Th1 (Kapila et al., 2013; Cai et al., 2016), which could also be an explanation of the increase in this study, and it would also be coherent with the raise of IFN-γ.

Flow cytometry analysis of immune cell populations isolated from mesenteric lymph nodes and Payer's patches in mice showed that the pre-treatment with antibiotics and *E. coli* lower the activity of $CD4^+$ and $CD8^+$ cells (Fig. 2A and B), which has previously been found after antibiotic treatment (Ekmekciu et al., 2017). The activity was restored to normal levels by the addition of *B. coagulans* to group TB1 and TB2, while *P. cedrina* and *R. cerastii* added to group TP1 and TR did not seem to have the same effect, rather the opposite. The reason is probably due to divergent interactions of the immune system and the microbiota, whose composition was found to vary between the treatment groups (Tables 2 and 3).

The dissimilarly induced immune reactions are probably due to different interactions between the microbiota and the immune system, supported by variations in the T-RF patterns between the treatment groups.

TLR2 recognizes Gram-positive bacteria, but have also been seen to play a role in Gram-negative infections (Spiller et al., 2008), explaining the larger population after treatment with antibiotics and *E. coli*

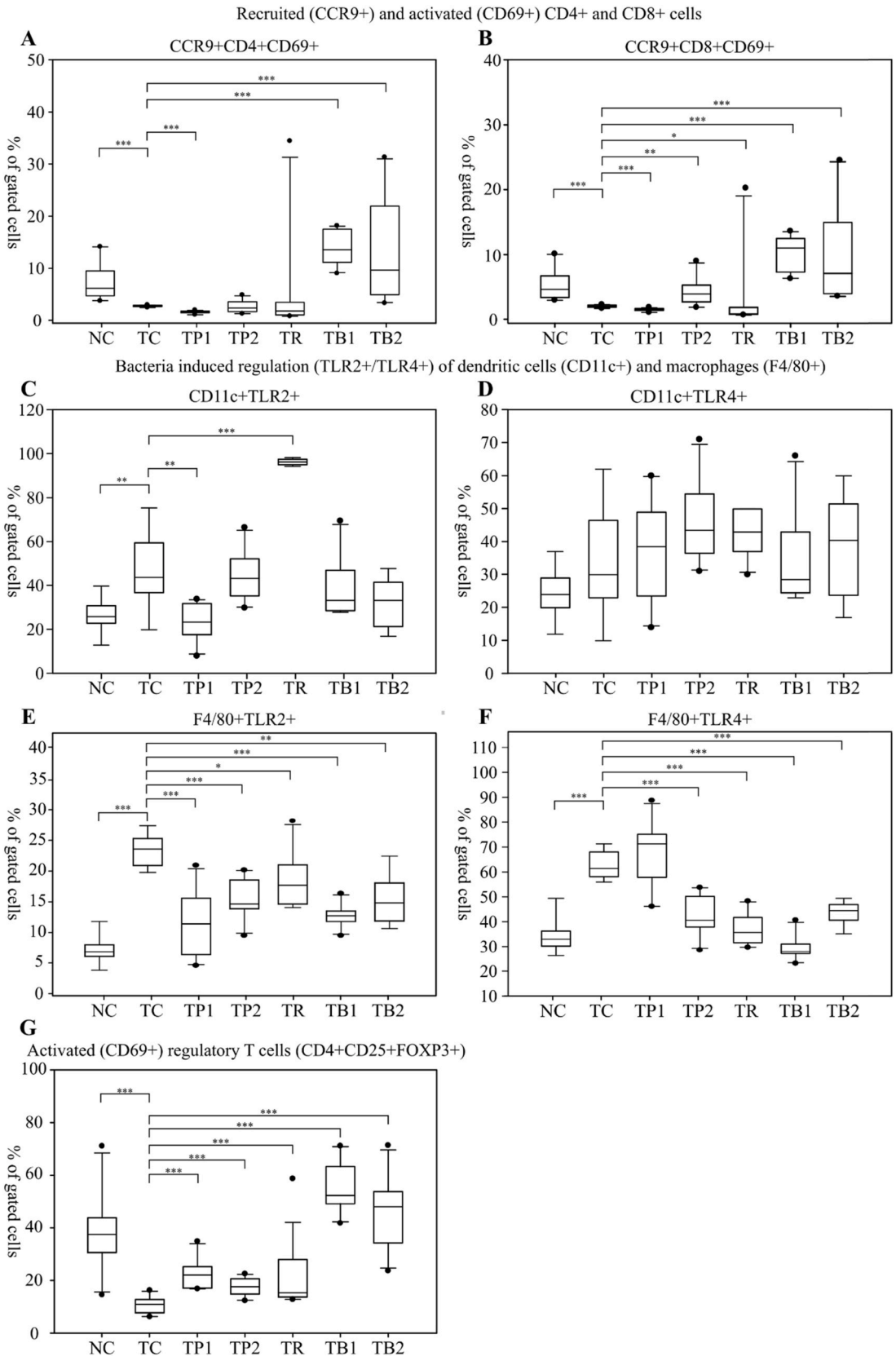

Fig. 2. Immune cell populations of mesenteric lymph nodes (MLN) and Peyers' patches (PPs) analyzed by flow cytometry. A-B shows results for MLN, C–F for PPs and G for MLN. Statistical differences between groups were compared with Kruskal-Wallis one-way ANOVA on ranks, and differences between two experimental groups were assessed by Mann-Whitney rank sum test. *P < 0.05, **P < 0.01 and ***P < 0.001 compared to treatment control (TC). NC: normal control, TC: treatment control, TP1: *P. cedrina* LMG P-32207, TP2: *P. punonensis* LMG P-32204, TR: *R. cerastii* MR5x, TB1: *B. coagulans* LMG P-32205, TB2: *B. coagulans* LMG P-32206.

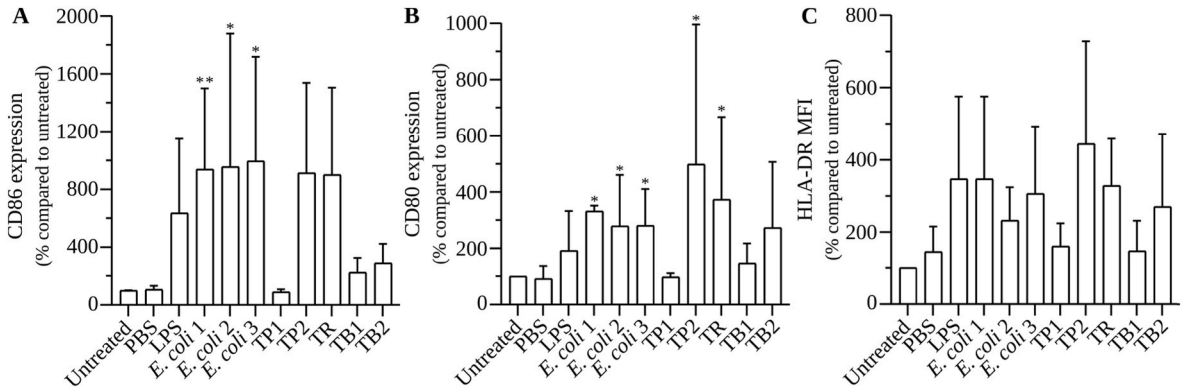

Fig. 3. Activation marker expression on MoDCs (n = 5) investigated by flow cytometry after 24 h of stimulation with indicated bacteria preparations. Statistical calculations were performed using Friedman's test. *P < 0.05 and **P < 0.01 compared to PBS. CD86 and CD80 data shown as mean % of positive cells in treated sample/mean % of positive cells in untreated sample. HLA-DR data shown as median fluorescence intensity (MFI) mean % of y-median of sample/mean % of y-median of untreated. Error bars represent standard deviation. *E. coli* 1: *E. coli* CCUG29300^T, *E. coli* 2–3: *E. coli* 2 and 3: wild strains. NC: normal control, TC: treatment control, TP1: *P. cedrina* LMG P-32207, TP2: *P. punonensis* LMG P-32204, TR: *R. cerastii* MR5x, TB1: *B. coagulans* LMG P-32205, TB2: *B. coagulans* LMG P-32206.

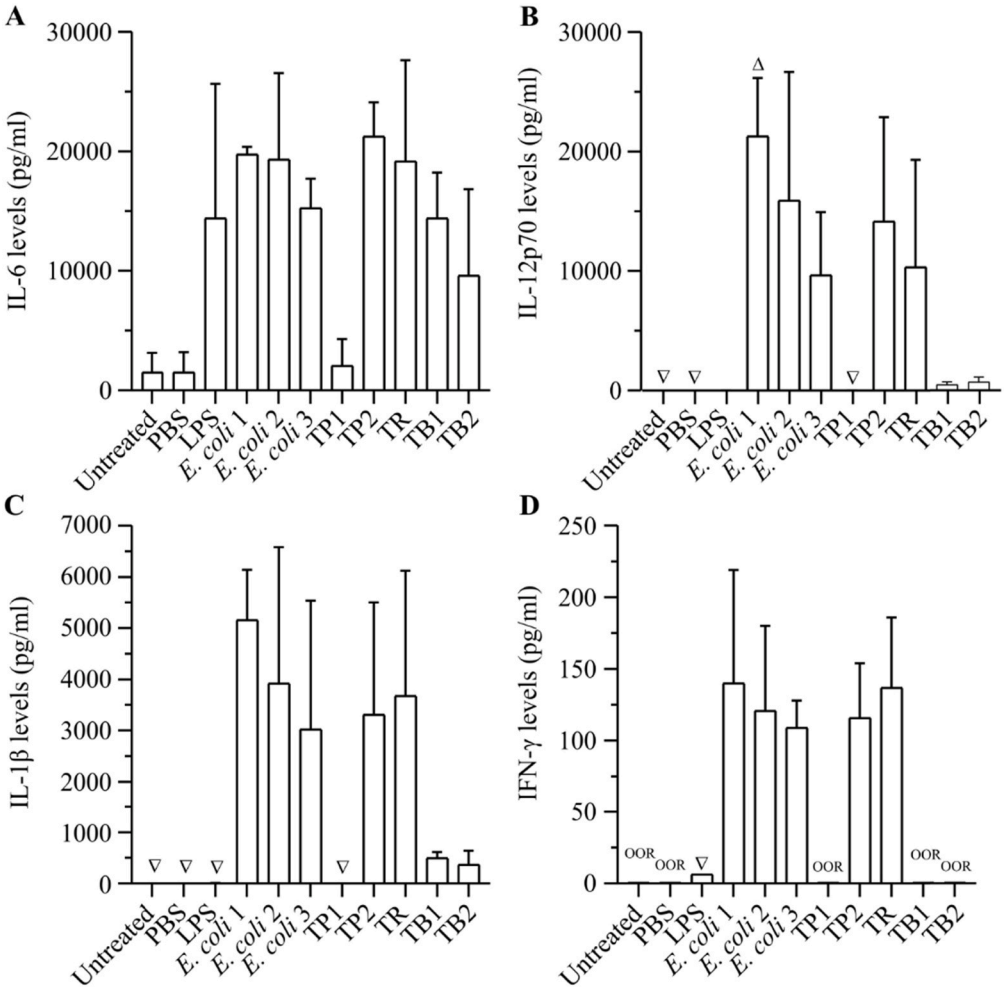

Fig. 4. Cytokine expression (please note different scaling) by MoDCs (n = 3, in technical duplicates) stimulated for 24 h with the indicated bacterial preparations in a 10:1 bacteria-to-MoDC ratio. Due to values out of range and high variation, no statistical evaluation was performed ($n_{(shown)}$ = 2–6). △ indicates one or more replicates out of range on the upper limit, ▽ indicates one or more replicates out of range on the lower limit. Error bars represent standard deviation. OOR – all replicates measured are below detection limit. *E. coli* 1: *E. coli* CCUG29300^T, *E. coli* 2 and 3: wild strains. NC: normal control, TC: treatment control, TP1: P. *cedrina* LMG P-32207, TP2: *P. punonensis* LMG P-32204, TR: *R. cerastii* MR5x, TB1: *B. coagulans* LMG P-32205, TB2: *B. coagulans* LMG P-32206.

compared to mice without treatment in this study. The levels of TLR2+ were restored towards normal levels in macrophages in all treatment groups, but in DCs for *R. cerastii* (TR), the effect was the opposite, indicating an induced immune response.

The stimulation by lipopolysaccharides (LPS) found in the outer membrane of Gram-negative bacteria is signaled through TLR4 (Munford and Varley, 2006). In the present study, the population of TLR4+ macrophages increased after treatment with *E. coli* (Fig. 2), an effect which was reduced by all added test strains except *P. cedrina*.

All activated regulatory T cell (CD_4+CD_{25}+$FoxP_3$+) populations were larger compared to treatment control, (Fig. 2G), especially for *B. coagulans* strains, indicating a stronger down regulation of the immune response. Since regulatory T cells produce IFN- γ, this result is in line with the observed raise of this cytokine (Wood and Sawitzki, 2006).

It has previously been documented that probiotic species are able to increase the population of regulatory T cells in the same way (Liu et al., 2010; Zhang et al., 2017; Groeger et al., 2013). Increased activity in regulatory T cells might have been induced by elevated levels of IFN-γ found in groups receiving *B. coagulans*, to create an anti-inflammatory effect.

T-RFLP is a reproducible technique suitable for comparing predominant community structures of the intestinal microbiota and calculate microbial diversity. Administration of the test strains fundamentally changed the microbiota in specific ways according to the administrated test strains, as seen in the occurrence of many different T-RFs. The changes in the microbiota could also be seen as an increase of Shannon-Wiener diversity index for group TP2 receiving *P. pununensis* in the small intestine, and for group TB2 (*B. coagulans*) in the large intestine.

In an approach to predict possible pro-inflammatory effects of these bacteria in humans, the same test strains evaluated in the *in vivo* model were inactivated, added to MoDCs, and the MoDC response was analyzed by flow cytometry and a multiplex cytokine assay.

The relative increase of CD86, CD80 and HLA-DR levels as measured by flow cytometry (Fig. 3) indicate MoDC activation in response to all *E. coli* strains, *P. pununensis* (TP2) and *R. cerastii* (TR) strains, which could be consistent with a pro-inflammatory response. The multiplex cytokine screening (Fig. 4) confirmed the pattern seen in the flow cytometry analysis: *P. pununensis* (TP2) and *R. cerastii* (TR), as well as all *E. coli* strains, caused MoDCs to produce a higher amount of pro-inflammatory cytokines IL-12p70, IL-1β and IFN-γ, supporting an overall pro-inflammatory response. IL-6 secretion seemed to be induced by all *E. coli* strains, *P. pununensis*, *R. cerastii* and also by the two *B. coagulans* strains (TB1 and TB2).

To the author's knowledge, there is no data available about the capability of *P. cedrina*, *P. pununensis*, and *R. cerastii* to activate human immune cells. However, heat-inactivated *B. coagulans* have been described to activate human PBMCs and to induce production of cytokines including IL-6, IL-12p70, IL-1β, and IFN-γ (Jensen et al., 2017). In the present study, only IL-6 increased by stimulation of *B. coagulans*, suggesting a strain specific response. Another publication summarizes safety assessments of a proprietary preparation of *B. coagulans*, GanedenBC30™, and concludes that "GanedenBC30TM is considered safe for chronic human consumption" (Endres et al., 2009). However, it is challenging to compare different studies due to varying and in part insufficiently described approaches how live or inactivated bacterial preparations were used. It seems clear that different approaches come with advantages and disadvantages, e.g. due to different degrees of structural integrity. Interestingly, a recent study comparing the capability of *Staphyloccocus aureus* as USA300 live strain, inactivated by heat, ultraviolet irradiation, or by paraformaldehyde treatment to stimulate human MoDCs. Similar MoDC viability and maturation marker expression was described upon exposure to the different bacteria preparations, but clear differences in cytokine expression were noted (Cruciani et al., 2019). In addition, the use of primary cells offers *in vivo* relevance but inherently also represents individual differences in the cellular response, which can lead to high variation.

To conclude, the immunomodulatory effects of the test strains in mice indicate that group TB1 and TB2 receiving *B. coagulans* strains consistently resemble the normal control, indicating a recovery from the pretreatment with antibiotics and *E. coli* and a less affected immune system. This is in line with the results obtained *in vitro* using human MoDCs, where absence or only a low increase of DC activation marker expression and pro-inflammatory cytokine expression was observed. On the other hand, groups TP1 and TR receiving *P. cedrina* and *R. cerastii* respectively, indicate an increase in inflammatory status. The results obtained after treatment of human MoDCs with *R. cerastii* support this finding, which is not the case for *P. cedrina*. The *P. pununensis* strain seems to have weaker influence on the immune system that differs from *P. cedrina*, indicating a bacterial species-specific response.

The two strains of *B. coagulans* showed an immunological response that indicate that these two test strains lack pathogenic abilities. In contrary, they even seem to generate beneficial effects in a probiotic fashion. This should qualify them to be included in safety tests according to national guidelines involving antibiotic susceptibility tests, biogenic amine production and toxin production (Laulund et al., 2017; Ferreira et al., 2019). This study shows great initial potential for *B. coagulans* to be used as biocontrol agents and enhance the safety on leafy green vegetables.

CRediT authorship contribution statement

Elisabeth Uhlig: Formal analysis, Investigation, Data curation, Writing – original draft, preparation, Writing – review & editing, Visualization, All authors have read and agreed to the published version of the manuscript. **Giulia Elli:** Investigation, Data curation, Writing – original draft, preparation, Visualization, All authors have read and agreed to the published version of the manuscript. **Noora Nurminen:** Investigation, All authors have read and agreed to the published version of the manuscript. **Elin Oscarsson:** Investigation, All authors have read and agreed to the published version of the manuscript. **Pamela Canaviri-Paz:** Investigation, All authors have read and agreed to the published version of the manuscript. **Stina Burri:** Investigation, All authors have read and agreed to the published version of the manuscript. **Anne-Marie Rohrstock:** Methodology, Supervision, All authors have read and agreed to the published version of the manuscript. **Milladur Rahman:** Methodology, Software, Validation, Supervision, All authors have read and agreed to the published version of the manuscript. **Beatrix Alsanius:** Conceptualization, Funding acquisition, All authors have read and agreed to the published version of the manuscript. **Göran Molin:** Writing – review & editing, All authors have read and agreed to the published version of the manuscript. **Kathrin Stephanie Zeller:** Conceptualization, Methodology, Validation, Investigation, Data curation, Writing – original draft, preparation, Writing – review & editing, Visualization, Project administration, Funding acquisition, All authors have read and agreed to the published version of the manuscript. **Åsa Håkansson:** Conceptualization, Methodology, Validation, Investigation, Writing – review & editing, Supervision, Project administration, Funding acquisition, All authors have read and agreed to the published version of the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.fct.2022.113064.

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