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Comparison of eight *Lactobacillus* species for delivery of surface-displayed mycobacterial antigen

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ABSTRACT

Lactobacillus spp. comprise a large group of Gram-positive lactic acid bacteria with varying physiological, ecological and immunomodulatory properties that are widely exploited by mankind, primarily in food production and as health-promoting probiotics. Recent years have shown increased interest in using lactobacilli for delivery of vaccines, mainly due to their ability to skew the immune system towards pro-inflammatory responses. We have compared the potential of eight *Lactobacillus* species, *L. plantarum*, *L. brevis*, *L. curvatus*, *L. rhamnosus*, *L. sakei*, *L. gasseri*, *L. acidophilus* and *L. reuteri*, as immunogenic carriers of the Ag85B-ESAT-6 antigen from *Mycobacterium tuberculosis*. Surface-display of the antigen was achieved in *L. plantarum, L. brevis, L. gasseri* and *L. reuteri* and these strains were further analyzed in terms of their *in vitro* and *in vivo* immunogenicity. All strains activated human dendritic cells *in vitro*. Immunization of mice using a homologous prime-boost regimen comprising a primary subcutaneous immunization followed by three intranasal boosters, led to slightly elevated IgG levels in serum in most strains, and, importantly, to significantly increased levels of antigen-specific mucosal IgA. Cellular immunity was assessed by studying antigen-specific T cell responses in splenocytes, which did not reveal proliferation as assessed by the expression of Ki67, but which showed clear antigen-specific IFN-γ and IL-17 responses for some of the groups. Taken together, the present results indicate that *L. plantarum* and *L. brevis* are the most promising carriers of TB vaccines.

1. Introduction

Lactobacillus spp. comprise a large heterogeneous group of Gram-positive, non-sporulating lactic acid bacteria (LAB) that produce lactic acid as a metabolite of sugar fermentation [1]. The genus *Lactobacillus* consists of more than 180 species that differ in their biochemical, ecological, molecular and immunomodulatory properties [2,3]. LAB inhabit various ecological niches including meat, milk and plants, as well as vaginal cavities and the gastrointestinal (GI) tracts of humans and animals [1]. Numerous lactobacilli are exploited in food production, where they contribute to taste and texture, while having a preservative effect due to acidification and production of bacteriocins [4]. Several *Lactobacillus* species have the Generally Regarded as Safe (GRAS) status, including strains that are recognized as probiotics, meaning that they have health-promoting effects on the host. Beneficial properties of lactobacilli are thought to relate to their ability to maintain intestinal homeostasis by modulating the intestinal microbiota, inhibiting pathogen growth, and/or controlling intestinal permeability [2].

Besides these industrial and probiotic applications, recent years have shown increased interest in members of the genus *Lactobacillus* as carriers for the delivery of vaccines, in particular vaccines administered through mucosal routes [2]. The possibility to make lactobacilli express heterologous antigens that become located in various cellular locations (cytoplasmic, secreted or associated to cell surface) is well documented [2,5,6]. Some *Lactobacillus* species effectively adhere to epithelial cells and colonize host cavities [7], which results in longer persistence at mucosal sites and thereby prolongs exposure of the mucosal immune system to a carried vaccine. Moreover, lactobacilli are characterized by their ability for interacting with innate immune cells and for modulating the immune system and are thus considered to display natural adjuvanticity [8]. *Lactobacillus* spp. modulating immune system towards pro-inflammatory responses are of particular interest in vaccine development, as pro-inflammatory properties may enhance desirable responses to a carried antigen. Notably, numerous studies have shown that the type of immune responses evoked by lactobacilli varies between the species or even strains [3,9-12]

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and these observations emphasize that the selection of a proper carrier strain for a vaccine remains of high importance.

Lactobacillus showing pro-inflammatory effects in *in vitro* analyses include species such as *L. gasseri*, *L. reuteri*, *L. jansoni*, *L. casei* or *L. plantarum* [9,10,13]. Notably, Ibnou-Zekri et al. have demonstrated that two intestinal lactobacilli, despite showing similar properties *in vitro*, induced different immune responses upon oral administration in mice [11], thereby highlighting the need for *in vivo* studies. Animal studies have shown immunogenicity or protective efficacy of several lactobacilli carrying vaccines against viral or bacterial infectious diseases, including *L. casei* [14], *L. acidophilus* [15], *L. brevis* [12] and *L. plantarum* [16]. *L. casei* has also been tested as a potential carrier for a therapeutic vaccine against HPV and elicited specific immunity in human objects [17].

In the current study, we have assessed various lactobacilli as candidate carriers for a vaccine against tuberculosis (TB). TB, caused by *Mycobacterium tuberculosis*, remains one of the most deadly diseases in the world. The only licensed vaccine against TB, the Bacillus Calmette-Guérin (BCG) vaccine, can be highly protective but has a number of limitations, such as deficient efficacy in adults and undesirable effects in HIV-infected persons. Therefore, the development of a new vaccine that will effectively prevent spreading of *M. tuberculosis* infections is a worldwide urgency. Platforms that are being developed for carrying TB antigens include attenuated pathogenic bacteria (e.g. live-modified *M. tuberculosis* itself [18]), non-pathogenic bacteria (e.g. *Bacillus subtilis* spores [19]), non-replicating viruses (e.g. Ankara virus [20]) and nanoparticles [21]. The strategies are aimed not only at delivering vaccines in stable from, but also to provide adjuvant effects that boost the desired immune responses.

We have previously shown that *Lactobacillus plantarum* can produce surface-attached *M. tuberculosis* fusion antigens and, more importantly, that antigen-displaying strains induce antigen-specific responses after oral or intranasal immunization in mice [22], and offer protection against *M. tuberculosis* infection [23]. The goal of the present study was to examine eight *Lactobacillus* species as potential vectors for a TB vaccine. *L. plantarum*, *L. brevis*, *L. curvatus*, *L. rhamnosus*, *L. sakei*, *L. gasseri*, *L. acidophilus* and *L. reuteri* were engineered to produce a fusion protein comprised of the Ag85B and ESAT-6 antigens (referred to as AgE6) of *M. tuberculosis* and to anchor this protein to the bacterial surface via an N-terminal covalent lipoprotein anchor [6,22]. We used this AgE6 fusion protein [22], which is highly similar to the well-known H1 antigen [24] to allow comparison with earlier studies using this model antigen and because H1 has shown promising results in pre-clinical as well as human trials. In recent years, the Ag85B-ESAT fusion antigens have been surpassed by H56, which contains a third antigen called Rv2660c [25,26]. The engineered strains were characterized in terms of growth, as well as production and surface display of the antigen. The most promising candidates, *L. brevis*, *L. reuteri*, *L. gasseri* and *L. plantarum*, were then tested for activation of human dendritic cells (DCs) *in vitro* and used in a mouse model study to assess and compare their immunogenicity *in vivo*.

2. Materials and methods

2.1. Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *Lactobacillus* species were cultured in MRS broth (Oxoid Ltd., Basingstoke, United Kingdom) without shaking at 37 °C (*L. plantarum*, *L. gasseri*, *L. reuteri*, *L. acidophilus* and *L. rhamnosus*) or 30 °C (*L. sakei*, *L. curvatus* and *L. brevis*). *L. lactis* was grown in M17 (Oxoid) medium supplemented with 0.5% (w/v) glucose (GM17) at 30 °C without shaking. Erythromycin was added to a final concentration of 10 μg/ml. Liquid medium was solidified by adding 1.5% (w/v) agar.

2.2. DNA manipulations

The final expression vector pLp_1261AE6-DC_SH71 is a derivative of pLp_1261AgE6-DC [22], in which the original 256_{rep} replicon is replaced by the broad host and higher copy number $SH71_{rep}$ replicon [27]. To make this change, plasmid pSIP411 [27] was digested with *Bsa*I and *Hind*III restriction enzymes. The resulting 3093 bp DNA fragment, encoding $SH71_{rep}$, was cloned into *Bsa*I/

Table 1
Plasmids and strains used in this study.

Strain or plasmid	Description	Reference
Plasmids		
pSIP411	Ery^r; pSIP401 derivative harboring the broad spectrum SH71 replicon	[27]
pLp_1261AgE6-DC	Ery^r; plasmid encoding the AgE6 antigen fused to an N-terminal lipo-anchor, and harboring the narrow host replicon 256	[22]
pEV	Ery^r; control plasmid (“empty vector”)	[6]
pLp_1261AE6-DC_SH71	Ery^r; pLp_1261AgE6-DC derivative, where the 256 replicon has been replaced by the SH71 replicon	This study
Strains		
Lactobacillus plantarum WCFS1	Host strain	[49]
Lactococcus lactis IL 1403	Host strain	[50]
Lactobacillus gasseri ATCC 33323^T	Host strain	[51]
Lactobacillus reuteri DSM 20016	Host strain	DSMZ
Lactobacillus acidophilus ATCC 4356	Host strain	ATCC
Lactobacillus sakei Lb790	Host strain	[52]
Lactobacillus rhamnosus GG	Host strain	Valio Ltd, Finland [53]
Lactobacillus curvatus DSM 20019	Host strain	DSMZ
Lactobacillus brevis DSM 20556	Host strain	DSMZ
L_plantarum-AgE6	*L. plantarum* harboring pLp_1261AE6-DC_SH71	This study
L_gasseri-AgE6	*L. gasseri* harboring pLp_1261AE6-DC_SH71	This study
L_reuteri-AgE6	*L. reuteri* harboring pLp_1261AE6-DC_SH71	This study
L_acidophilus-AgE6	*L. acidophilus* harboring pLp_1261AE6-DC_SH71	This study
L_sakei-AgE6	*L. sakei* harboring pLp_1261AE6-DC_SH71	This study
L_rhamnosus-AgE6	*L. rhamnosus* harboring pLp_1261AE6-DC_SH71	This study
L_curvatus-AgE6	*L. curvatus* harboring pLp_1261AE6-DC_SH71	This study
L_brevis-AgE6	*L. brevis* harboring pLp_1261AE6-DC_SH71	This study

*Hind*III digested pLp_1261AgE6-DC, yielding the pLp_1261AE6-DC_SH71. The vector was first transformed into *L. lactis* according to the method described by Holo and Nes [28] and positive transfromants were verified by DNA sequencing. Plasmid pLp_1261AE6-DC_SH71 was purified from *L. lactis* using NucleoSpin® Plasmid purification kit (Macherey-Nagel) and electroporated into the various *Lactobacillus* species. *L. reuteri* were electroporated according to Ahrné et al. [29], while the other *Lactobacillus* were electroporated as described elsewhere [30,31].

2.3. Growth, protein production and storage of cells

The expression of recombinant protein was induced and bacterial cells were harvested 3 h after induction, as described elsewhere [22,32]. The recombinant strains were inactivated by UV-irradiation for 1 h. Pellets of inactivated bacteria were stored at −80 °C until use. In order to determine the number of CFU, some of the freshly harvested bacterial cells were cultivated on solid MRS medium supplemented with antibiotics for 48 h and the colonies were counted. The numbers of inactivated bacteria were verified after storage at −80 °C by counting in a Buerker counting chamber (Paul Marienfeld GmbH & Co. KG, Germany).

2.4. Expression and surface localization of AgE6 antigen in Lactobacillus strains

Production of the AgE6 protein was analyzed as described in detail before [22,32]. Briefly, bacterial cells were harvested 3 h after induction. To analyze total AgE6 expression, bacterial cells were disrupted in FastPrep tubes containing 1.5 g of glass beads (size ≤ 106 μm; Sigma-Aldrich), using a FastPrep® FP120 Cell Disrupter with a shaking speed of 6.5 m/s, for 45 s. Cell debris was removed by centrifugation and crude cell-free protein extracts were subjected to Western blotting using a monoclonal mouse anti-ESAT-6 antibody and polyclonal HRP-conjugated anti-mouse IgG, as described before [22]. Surface localization of the AgE6 antigen was verified using flow cytometry and fluorescent microscopy of bacterial cells probed with a mouse anti-ESAT-6 specific antibody followed by staining with FITC-conjugated anti-mouse IgG, as described previously [22].

2.5. Isolation of human dendritic cells (DCs)

Human peripheral blood mononuclear cells were isolated and handled according to institutional ethical guidelines (Østfold Hospital Trust, Norway) and as described previously [22]. Briefly, cells were isolated by density gradient centrifugation for 25 min at 1500*g* using Lymphoprep TM (Axis-Shield Diagnostics Ltd., Dundee, Scotland) at room temperature and washed four times with PBS to remove the platelets. $CD14^+$ cells were separated on LS column (Miltenyi Biotec) using human CD14 MicroBeads (Miltenyi Biotec). Isolated $CD14^+$ cells (1×10^6) were seeded in 24-well plates and maintained in complete RPMI medium (RPMI-1640 containing 10% fetal bovine serum FBS, 1% penicillin-streptomycin, 2 mM L-glutamine, and 50 μM 2-mercaptoethanol, all from Sigma-Aldrich) supplemented with 25 ng/mL rhIL-4 and 50 ng/ml rhGM-CSF (ImmunoTools GmbH) for four days in a humidified incubator at 37 °C and 5% CO2 after which the medium was replaced with fresh IL-4- and GM-CSF-supplemented complete RPMI medium followed by cultivation for another three days.

2.6. Activation of human DCs by UV-inactivated Lactobacillus spp

1×10^6 DCs were incubated with *Lactobacillus* cells at a multiplicity of infection (MOI) of 200 for 48 h. As a positive control, a cocktail of 100 ng/ml LPS, 15 ng/ml TNF-α (ImmunoTools) and 5 μM PGE-2 (Sigma Aldrich) in complete RPMI medium was used. Cells from different blood donors were used, depending on availability during the course of the study. Stimulated cells were detached with trypsin (Biowest), transferred to a V-bottom 96-well plate and pre-incubated with human FcR Blocking Reagent (Miltenyi Biotec) diluted 1:50 in flow cytometry buffer containing 0.5% BSA (Sigma Aldrich) and 2 mM EDTA, in order to block non-specific binding of immunoglobulin to the Fc receptors. Subsequently, the cells were stained with anti-human antibodies specific for cell surface molecules: VioBright FITC-conjugated CD40 diluted 1:50 and PE-conjugated CD80, APC-conjugated CD83 and PE-conjugated HLA-DR diluted 1:11 (all from Miltenyi Biotec), for 20 min at 4 °C; and analyzed by flow cytometry using a MACSQuant analyzer (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), following the manufacturer's instructions. The data were analyzed and processed using FlowJo software.

2.7. Immunization protocol

All animal experiments were approved by the Norwegian Animal Research Authority (Mattilsynet, Norway). 6–8 weeks old female C57BL/6 BomTac mice were purchased from Taconic Bioscience (Ejby, Denmark). Mice were housed in pathogen-free conditions in individually ventilated cages (Innovive Inc, San Diego) under standard conditions (12 h light/dark cycle, 23–25 °C, 45–50% relative humidity). The mice were divided in five experimental groups (n = 8–9) immunized with different AgE6-producing *Lactobacillus*: *L. plantarum*, *L. gasseri*, *L. reuteri* and *L. brevis*, and naïve mice (non-immunized). A single immunization dose consisted of approximately $0.5–1 \times 10^9$ UV-inactivated bacterial cells and 20 μg the adjuvant poly(I:C) (Sigma-Aldrich), added shortly before immunization, in sterile PBS. Mice were immunized subcutaneously, followed by three intranasal boosting immunizations at two, four and six weeks after the start of the experiment and the volumes used were 100 μL for subcutaneous and 30–50 μL for intranasal immunization. The prime-boost strategy and adjuvant were chosen because this approach had shown promising results in a mouse challenge experiment with *Lactobacillus plantarum* expressing a surface-attached *M. tuberculosis* fusion antigen [23]. The experiment was terminated 3 weeks after the last immunization. Animals were euthanized by cervical dislocation under anesthesia. The anesthetic cocktail consisted in a mixture of Zoletil Forte (Virbac), Rompun (Bayer) and Fentadon (Eurovet) with the active substances Zolezepam (32 mg/kg), Tiletamin (32 mg/kg), Xylazine (4.5 mg/kg), and Fentanyl (26 ug/kg). It was given by subcutaneous injection (10 μL/g mouse) and all together they work to sedate, relieve pain and let the animals enter in full anesthesia. All procedures have been refined to provide for maximum comfort and minimal stress to the animals.

2.8. Sample collection and processing.

2.8.1. Isolation of splenocytes

Spleens were collected, mashed through 70 μm Corning® cell strainers (Sigma-Aldrich) and centrifuged at 300 × *g* for 10 min at room temperature. The cell pellets were resuspended and incubated in Red Cell Lysis buffer (Sigma-Aldrich) for 5 min and washed with complete RPMI medium. Cells were maintained in complete RPMI medium in a humidified incubator at 37 °C and 5% CO_2.

2.8.2. Serum preparation

Blood collected from mice was allowed to clot at room temperature for 1 h, followed by centrifugation at 1000–2000 × *g* for 10 min at 4 °C. Serum was stored at −20 °C until analysis.

2.8.3. Bronchoalveolar lavage (BAL)

Lungs were washed with 1 mL sterile PBS and the washes were collected and stored at −20 °C until analysis.

2.8.4. IgG and IgA assays

ELISA was used to determine antigen-specific titers for IgG in serum and IgA in BAL samples. Microtiter plates were coated with 2.5–5 μg/ml Ag85B or ESAT-6 (Lionex GmbH, Braunschweig, Germany) and incubated overnight at room temperature followed by blocking with 1% BSA in PBS for 1 h at 37 °C. Serial dilutions of serum (10-fold) and BAL (2-fold) samples were applied to precoated plates followed by incubation for 1 h at 37 °C. Subsequently, HRP-conjugated anti-mouse IgA (1:1000 dilution) or anti-mouse IgG (1:6000 dilution) antibodies (both from Sigma Aldrich) were used for detection of IgA or IgG, respectively, by incubation for 1 h at 37 °C. OPD Substrate Tablets (Sigma Aldrich) were used for color development, following the manufacturer's instructions. The OD at 450 nm was measured after 15 min incubation at room temperature.

2.8.5. T Cell proliferation

Freshly isolated splenocytes were seeded in 96-well plates and stimulated with 5 μg/mL Ag85B antigen or 1 μg/mL ESAT-6 antigen. Studies with recall antigens typically use 5 μg/mL, but we had to use a lower concentration for ESAT-6, because toxicity effects were observed when using 5 μg/mL. As positive control, the cells were stimulated with 1 μg/mL α-CD3 (BioLegends, San Diego, CA). Antigen specific T cell proliferation was analyzed after 6 days of incubation with antigen. Cells were pre-incubated in PBS containing TruStain fcX™ Fc Receptor Blocking Solution (BioLegend) diluted 1:500, and eBioscience™ Fixable Viability Dye eFluor™ 780 diluted 1:1000 in order to block non-specific binding of immunoglobulins to the Fc receptors and to exclude dead cells, respectively. The cells were subsequently stained with 1:100 diluted Brilliant Violet 421™-conjugated CD90.2 antibody determining $CD3^+$ T lymphocytes (BioLegends). After staining, the cells were fixed using the eBioscience™ Foxp3/Transcription Factor Staining Buffer Set and permeabilized using eBioscience™ Permeabilization Buffer, according to the manufacturer's instructions. Subsequently, cells were intracellularly stained with 1:50 diluted APC-conjugated Ki67 (BioLegends) antibody and analyzed by flow cytometry.

2.8.6. Cytokine analysis

Murine IFN-γ and IL-17 were analyzed in culture supernatants from splenocytes stimulated with 5 μg/mL Ag85B antigen or 1 μg/mL ESAT-6 antigen for 6 days. The cytokines were quantified by ELISA using eBioscience Ready-Set-Go kits, following the manufacturer's instructions and plates were read using a Multiskan™ FC Microplate Photometer (Thermo Scientific™).

3. Results

3.1. Characteristics of lactobacilli producing the AgE6 antigen

Eight *Lactobacillus* species: *L. plantarum, L. brevis, L. curvatus, L. rhamnosus, L. sakei, L. gasseri, L. acidophilus* and *L. reuteri*, were engineered to produce recombinant AgE6 antigen attached to the bacterial surface through a covalent lipoprotein anchor [22]. Intracellular production of AgE6 was investigated by subjecting cell-free protein extracts from induced bacterial cultures to Western blotting, which revealed that all species, except *L. acidophilus*, produced the recombinant antigen in appreciable levels (Fig. 1A). Flow cytometry revealed the presence of AgE6 on the bacterial surface for four strains: *L. plantarum*, *L. reuteri*, *L. brevis* and *L. gasseri* (Fig. 1B). Consistently, surface display of the antigen was confirmed by indirect fluorescence microscopy showing emission of fluorescence only for these same four species (Fig. 1C). Since surface-display of the antigen was considered essential, only those engineered strains with AgE6 antigen exposed at the bacterial surface, namely *L_plantarum*-AgE6, *L_brevis*-AgE6, *L_gasseri*-AgE6 and

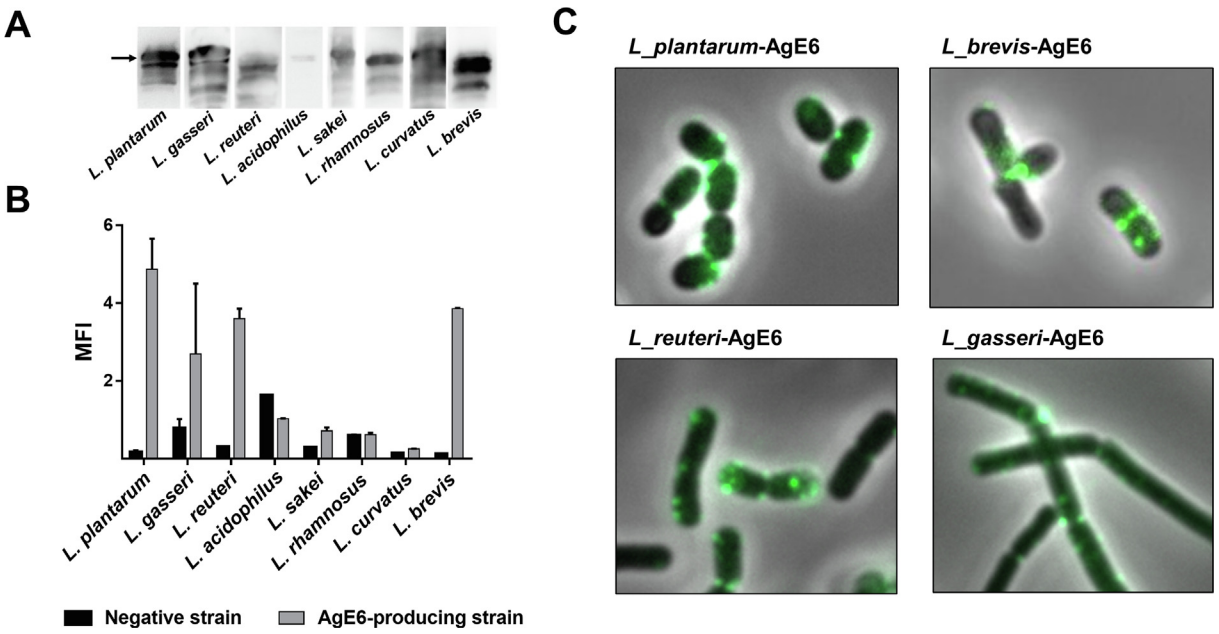

Fig. 1. Production and display of AgE6 on the surface of *Lactobacillus* spp. Panel A shows Western blots demonstrating intracellular production of the recombinant antigen in cell-free protein extracts of the eight tested strains. The approximate position of the intact fusion antigen (48 kDa) is indicated; in most cases multiple bands appear, which is common when using the Lp_1261 lipoanchor (22, 40) and likely results from proteolytic degradation. Panels B and C address surface display of the antigen. Bacterial cells from induced cultures were probed with a FITC-conjugated specific antibody. *Lactobacillus* strains harboring the empty vector, pEV, were used as negative controls. Panel B shows flow cytometry analysis and the results are presented as the medians of fluorescence intensity (MFI) for both AgE6-producing strains and the negative control strains. Panel C shows indirect immunofluorescence microscopy of the strains that appeared positive in the flow cytometry analysis displayed in panel B. The results presented are from three independent experiments and the data are shown as a mean ± SEM in panel B, whereas panels A and B show representative experiments.

L_reuteri-AgE6 (Fig. 1B and C), were subjected to subsequent studies.

3.2. Activation of dendritic cells

In the next step, we studied whether the four *Lactobacillus* spp. with AgE6 antigen on their surfaces were able to activate human dendritic cells (DCs), which are professional antigen-presenting cells (APCs). Activation of APCs plays a crucial role in T cell responses to the vaccine and is manifested in up-regulation of surface co-stimulatory molecules, so called maturation markers. We quantified expression of CD40, CD83, CD80 and HLA-DR maturation markers in DCs incubated with AgE6-producing lactobacilli. The results showed statistically significant up-regulation of CD40, CD80 and CD83 for pulsing with *L_plantarum*-AgE6 and *L_gasseri*-AgE6, as well as a positive tendency for HLA-DR (Fig. 2). *L_brevis*-AgE6 enhanced expression of CD40 and showed a tendency towards increased quantities of CD80, CD83 and HLA-DR. *L_reuteri*-AgE6 significantly up-regulated all four markers (Fig. 2).

3.3. Humoral immunity induced by AgE6-producing lactobacilli

We then assessed the immunogenic potential of the four AgE6-displaying *Lactobacillus* species in a mouse model, applying a homologous prime-boost strategy. The animals were primarily vaccinated via the subcutaneous route and subsequently given three intranasal boosters. The subcutaneous injection did not provoke any visible site reactions. Non-immunized mice (naïve group) were included as a negative control.

Analysis of serum samples indicated a trend for elevated IgG specific to Ag85B only for mice immunized with *L_plantarum*-AgE6 (Fig. 3A), whereas trends for elevated anti-ESAT-6 IgG were observed for all four groups of mice given antigen-producing lactobacilli (Fig. 3A). Analysis of mucosal antibodies in lung washes showed that *L_plantarum*-AgE6, *L_brevis*-AgE6 and *L_reuteri*-AgE6 generated significantly increased levels of both Ag85B-specific and ESAT-6-specific IgA, whereas immunization with *L_gasseri*-AgE6 showed a similar tendency. Thus, all tested AgE6-producing lactobacilli seemed to induce humoral immunity, albeit to varying extents.

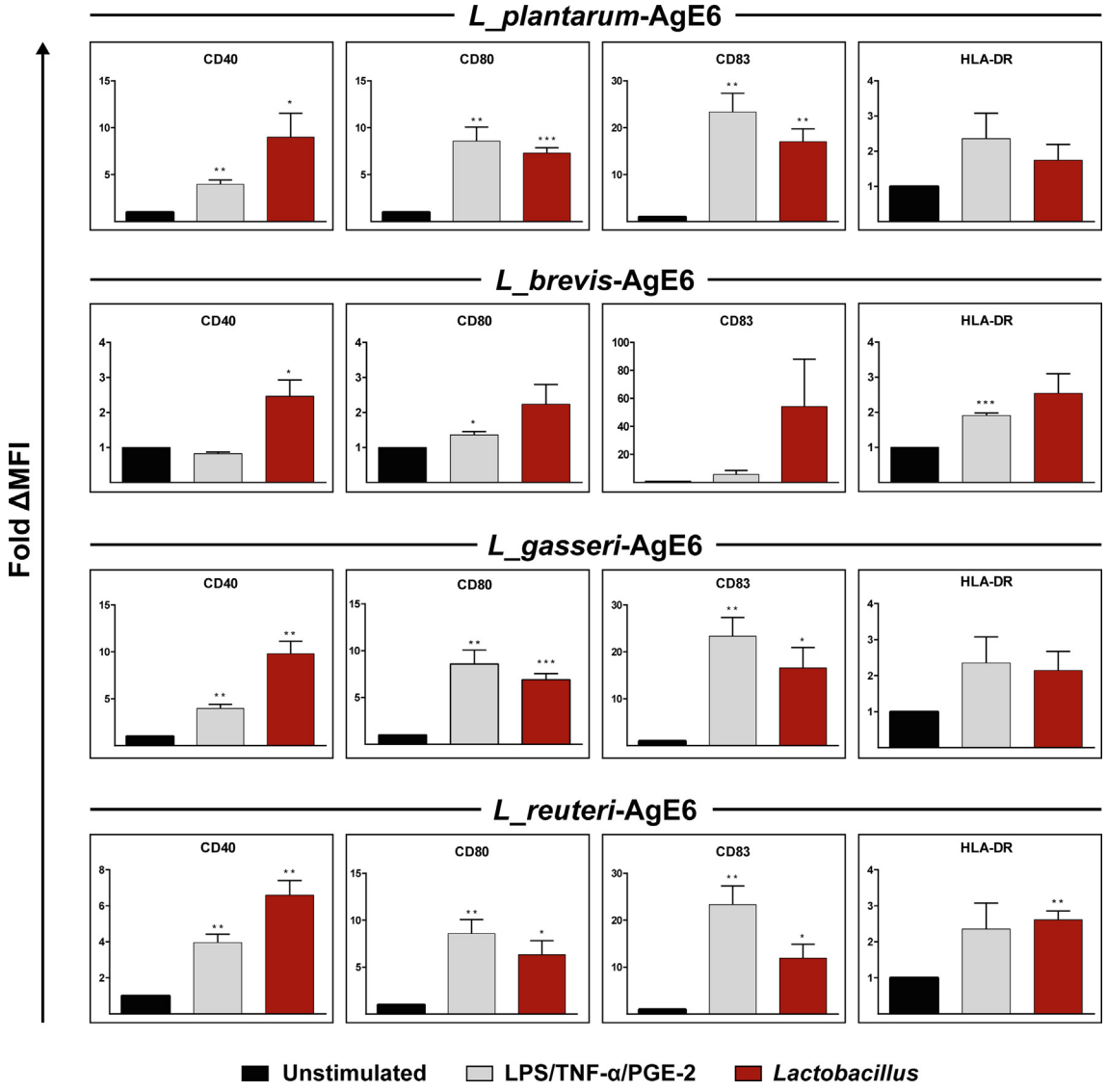

Fig. 2. Activation of DCs by recombinant *Lactobacillus* species. Human DCs were stimulated with *L_plantarum*-AgE6, *L_brevis*-AgE6, *L_gasseri*-AgE6 or *L_reuteri*-AgE6 for 48 h at an MOI of 200. A cocktail of LPS/TNF-α/PGE-2 was used as a positive control. Expression of surface co-stimulatory molecules CD40, CD80, CD83 and HLA-DR was measured by flow cytometry and the median of fluorescence intensity (MFI) was normalized to the MFI of the unstimulated control, which was set to 1. Each Lactobacillus was tested with cells from three blood donors and the data are shown as a mean ± SEM. Note that different donors were used for different lactobacilli; hence the MFI values needed to normalized to allow comparison between the different strains. Statistically significant differences relative to the unstimulated controls were determined using unpaired *t*-tests and are indicated as follows: $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

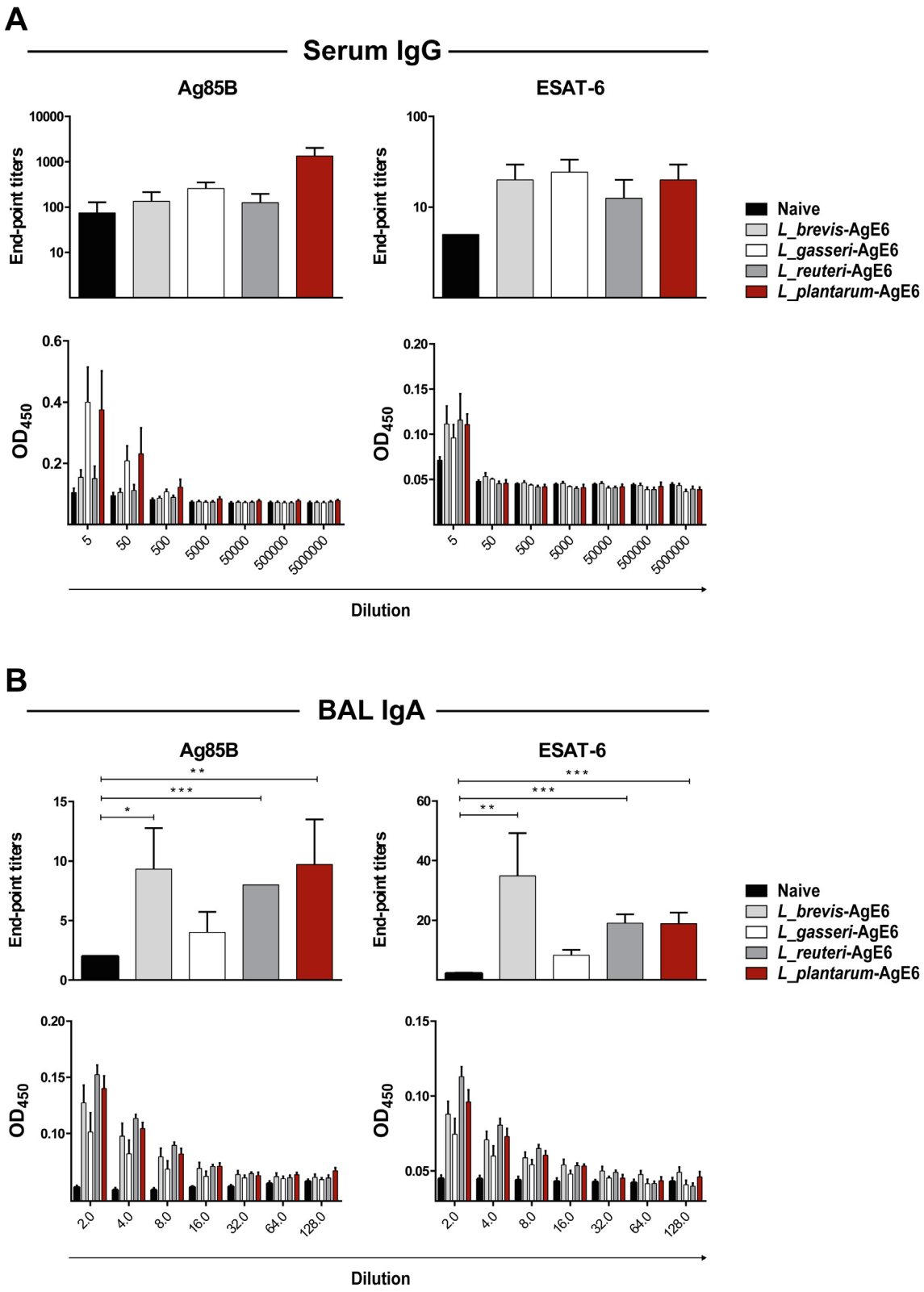

Fig. 3. Humoral responses induced by lactobacilli producing the AgE6 antigen. Serum (10-fold dilutions) and BAL (2-fold dilutions) samples were subjected to ELISA in order to determine antibodies specific to Ag85B or ESAT-6. The end point titers (upper panels) and serial dilutions (lower panels) were assessed for serum IgG (A) and BAL IgA (B). The results are presented as means ± SEM (n = 7-9). Statistically significant differences were determined using Kruskal-Wallis one-way ANOVA followed by Dunn's multiple comparison post-hoc test and are indicated as follows: $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

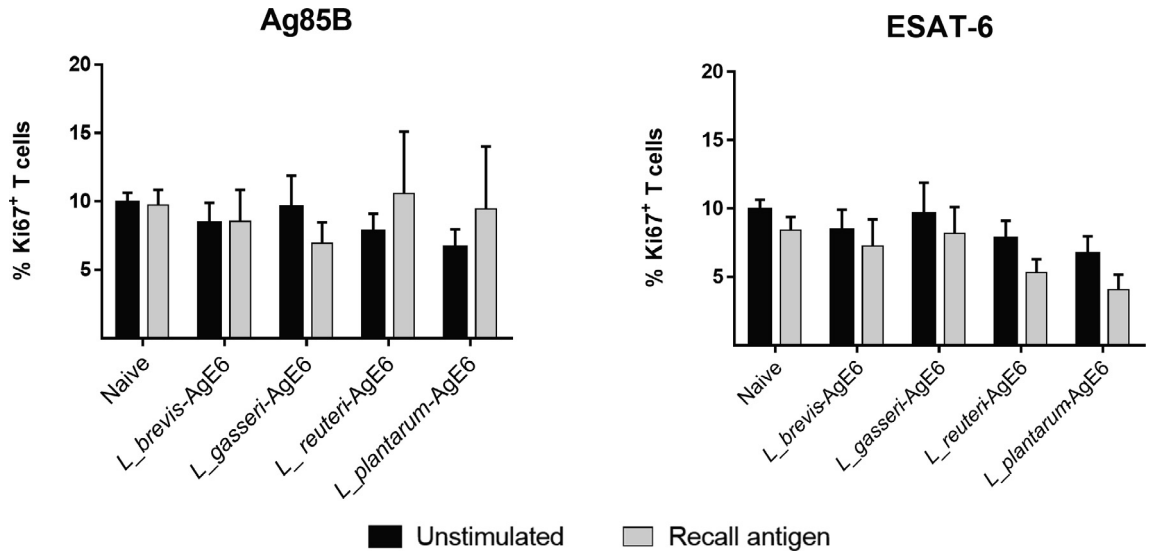

Fig. 4. Antigen specific T cell proliferation. Splenocytes from immunized mice were stimulated with Ag85B or ESAT-6 in technical duplicates for 6 days. T cell proliferation was analyzed by Ki67 staining using a single cells → live → $CD3^+$ → $Ki67^+$ gating strategy. The results are presented as a mean ± SEM ($n = 5$).

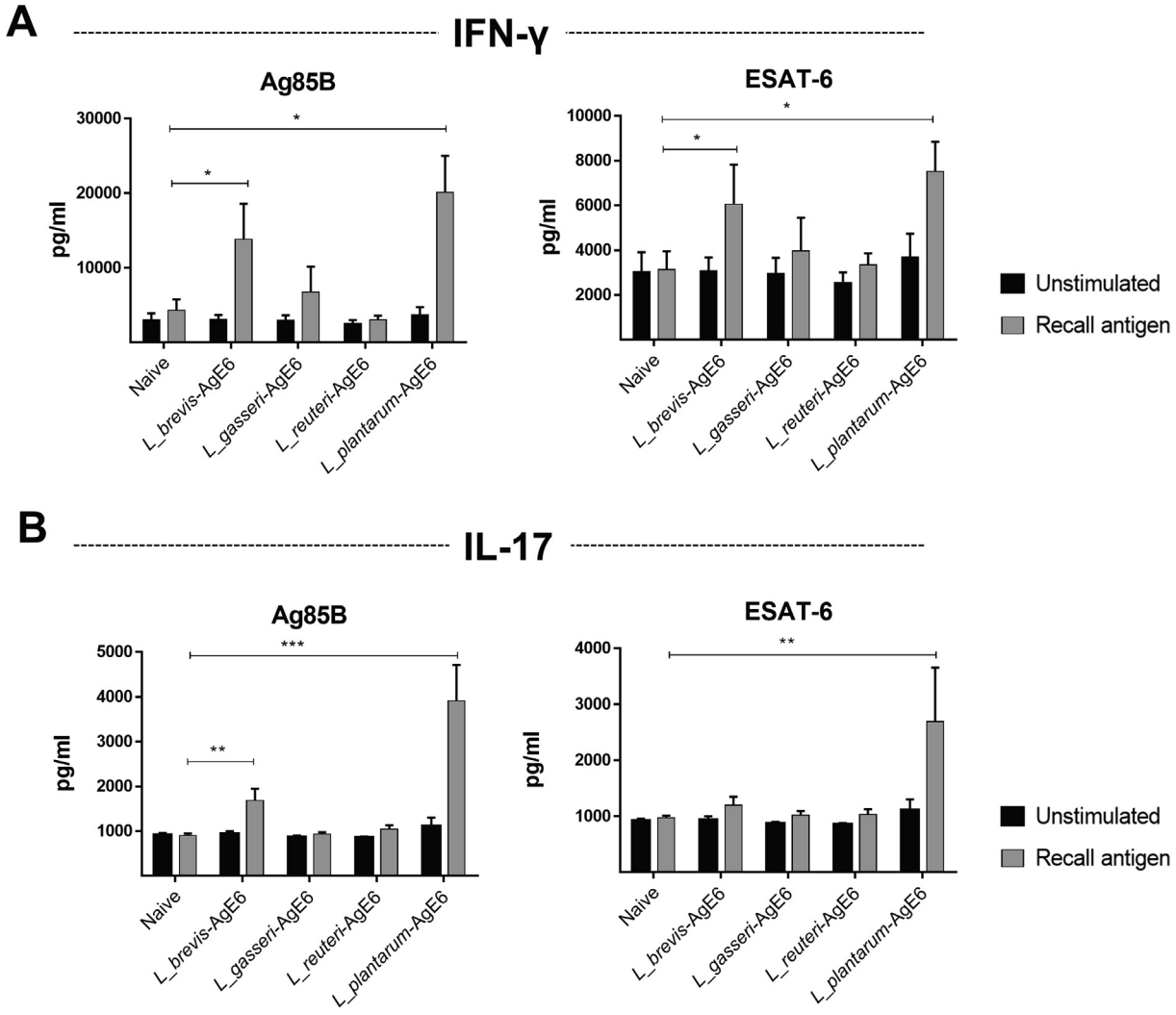

Fig. 5. Antigen-specific IFN-γ (A) and IL-17 (B) secretion recalled by antigens in proliferating splenic T cells. Splenocytes from immunized mice were stimulated with Ag85B or ESAT-6 for 6 days in technical duplicates and the levels of secreted IFN-γ (A) and IL-17 (B) in culture supernatants were quantified by ELISA. The results are presented as a mean ± SEM ($n = 8$). Statistically significant differences were determined using the Mann-Whitney U test and are indicated as follows: $^*p < 0.05$, $^{**}p < 0.01$ and $^{**}p < 0.001$.

3.4. Cellular immunity induced by AgE6-producing lactobacilli

To evaluate cellular immune responses, in a first step, we examined antigen-specific proliferation of splenic T cells from immunized mice, by measuring expression of the proliferation marker Ki67 (Fig. 4). A non-significant increase in the frequency of $Ki67^+$ cells was only noticeable upon stimulation with Ag85B and only for groups immunized with *L_plantarum*-AgE6 or *L_reuteri*-AgE6 (Fig. 4). Overall, the data indicate that proliferative effects were negligible.

Cellular immunity was also assessed by antigen-specific production of IFN-γ and IL-17 in splenocytes from immunized animals. Both cytokines are known to correlate with protective immunity during *M. tuberculosis* infections [33,34], and IFN-γ is commonly used as an indicator of protective immunity offered by TB vaccine candidates [33]. The results (Fig. 5) show that *L_plantarum*-AgE6 and *L_brevis*-AgE6 evoked significantly increased IFN-γ levels specific to Ag85B or ESAT-6 (Fig. 5A). *L_gasseri*-AgE6 also induced antigen-specific IFN-γ secretion, but the effects were non-significant (relative to the control group) and much weaker compared to *L_plantarum*-AgE6 or *L_brevis*-AgE6 (Fig. 5A).

IL-17 levels were clearly increased upon incubation of splenocytes isolated from mice given *L_plantarum*-AgE6 with each of the two recall antigens (Fig. 5B). For *L_brevis*-AgE6, an IL-17 response was observed upon incubation with Ag85B, while pulsing with ESAT-6 recalled inconsiderable IL-17 secretion (Fig. 5B). Splenocytes from mice immunized with *L_gasseri*-AgE6 or *L_reuteri*-AgE6 did not show antigen-specific IL-17 responses.

4. Discussion

Members of the genus *Lactobacillus* are increasingly explored as potential carriers for delivery of heterologous molecules of prophylactic or therapeutic nature [35]. Lactobacilli provide numerous benefits, such as safety, simple and low-cost production, post-engineering stability, non-invasive administration, adjuvant effects and the potential to induce mucosal immunity. Lactobacilli expressing foreign antigens have been proposed as vaccines against a variety of infectious diseases, including viral infections, e.g. HPV [36] or influenza virus [37], and bacterial infections, e.g. *Chlamydia trachomatis* [32] or *Bacillus anthracis* [38]. Remarkably, to our knowledge, there are only two studies on using *Lactobacillus* as a vehicle for *M. tuberculosis* antigens and both of these used *L. plantarum* [22,39].

Further development of *Lactobacillus*-based vaccines depends in part on finding the optimal bacterial species, since the immunogenic characters of lactobacilli differ. Building on earlier work on developing a TB vaccine based on *L. plantarum*, we therefore compared eight *Lactobacillus* species as potential carriers for the AgE6 antigen. All strains were engineered to display AgE6 on their surface by fusing the antigen to an N-terminal lipoprotein anchor that has previously been used successfully to display AgE6 [22] and several other heterologous molecules, such as adhesins [6], chemokines [40] or active enzymes [41], in *L. plantarum*. Intracellular production of AgE6 protein was detected in all recombinant lactobacilli, except *L_acidophilus*-AgE6, indicating that the inducible expression system worked in seven of the eight tested species. However, surface-located antigen was detected in only four of the seven AgE6 producers, namely *L_brevis*-AgE6, *L_gasseri*-AgE6, *L_reuteri*-AgE6 and *L_plantarum*-AgE6. Lack of detection of surface-located antigen may indicate inefficient secretion or a sub-optimal orientation of the reactive part of the displayed protein. Surface-attachment of the antigens can be important for inducing antibody-mediated immunity, because B cells are able to recognize and extract immobilized and unprocessed antigens [42]. Therefore, only the four species with surface-displayed AgE6 were assessed in subsequent studies.

Post-immunization priming of T cells is strictly related to activation of APCs [43]. Conserved components of bacterial cells, such as peptidoglycan or lipoteichoic acid, can activate innate immune cells, and the ability of lactobacilli to trigger maturation of dendritic cells is well known (e.g. [9,44]). Indeed, we demonstrated that the four recombinant AgE6-displaying lactobacilli induce up-regulation of the CD40, CD80, CD83 and HLA-DR co-stimulatory molecules and that the four species have similar effects (Fig. 2).

Control of *M. tuberculosis* infections requires humoral and, to a greater extent, cellular immunity [45]. As to humoral responses, all four AgE6-carrying lactobacilli induced mucosal and serum antibody responses, yet the observed effects varied. Two strains, namely *L. plantarum* and *L. brevis*, stand out by their relatively high immunogenic character. Next to humoral immunity, both *L_plantarum*-AgE6 and *L_brevis*-AgE6 evoked antigen-specific IFN-γ and IL-17 secretion in splenocytes from immunized mice, indicative of a cellular response. Although *L. gasseri* [38] and *L. reuteri* [46] have shown some success as antigen carriers, the present study indicates that they are relatively poor inducers of immune responses to mycobacterial antigens.

L. plantarum remains one of the most intensively exploited lactobacilli in vaccine development [2,35], mainly due to its proven adjuvanticity [47,48]. Remarkably, while *L. brevis* also has clear adjuvant properties [12], to our knowledge, this species has not been explored as a candidate for vaccine delivery. Here, we show that *L. brevis*, similarly to *L. plantarum*, is capable of generating anti-mycobacterial immunity in both mucosal and systemic compartments of the immune system.

To conclude, we show that lactobacilli have varying potentials for production and surface-display of a recombinant mycobacterial antigen when using the pSIP expression system. The functional studies of the four lactobacilli displaying the antigen show considerable variation and indicate that *L. brevis* is a promising alternative to *L. plantarum*, at least for this antigen and this expression system. At the same time, our study shows that much used *L. plantarum* indeed is among the very best *Lactobacillus* species for this purpose.

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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Authors' contributions

GM, KK and VE designed the study. LØ, SR and KK acquired the data. KK analyzed the data and drafted the paper. KK, GM and VE contributed to preparing the final version of the manuscript. All authors read and approved the final manuscript.

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