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Secretion and functional expression of *Mycobacterium bovis* antigens MPB70 and MPB83 in lactic acid bacteria

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ABSTRACT

The aim of this study was to determine the reliability of lactic acid bacteria (LAB) as heterologous hosts for the expression of MPB70 and MPB83, two *Mycobacterium bovis* antigens that possess diagnostics and immunogenic properties, respectively. We therefore generated recombinant cells of *Lactococcus lactis* and *Lactobacillus plantarum* that carried hybrid genes encoding MPB70 and MPB83 fused to signal peptides that are specifically recognized by LAB. Only *L. lactis* was able to secrete MPB70 using the *L. lactis* signal peptide Usp45, and to produce MPB83 as an immunogenic membrane protein following its expression with the signal peptide of the *L. plantarum* lipoprotein prsA. Inactivated cells of MPB83-expressing *L. lactis* cultures enhanced NF-κB activation in macrophages. Our results show that *L. lactis* is a reliable host for the secretion and functional expression of antigens that are naturally produced by *M. bovis*, the causative agent of bovine tuberculosis (bTB). This represents the first step on a long process to establishing whether recombinant LAB could serve as a food-grade platform for potential diagnostic tools and/or vaccine interventions for use against bTB, a chronic disease that primarily affects cattle but also humans and a wide range of domestic and wild animals.

1. Introduction

Lactic acid bacteria (LAB) are Gram-positive, non-pathogenic bacteria frequently isolated from food that possess a remarkable adaptability to technological and physiological stress conditions [1]. LAB also inhabit the gastrointestinal tract of animals, contributing to the maintenance of gut homeostasis due to their multiple health-promoting properties such as the production of compounds involved in beneficial immunomodulatory responses and antagonistic activity against opportunistic pathogens [2–4]. Therefore, LAB are ideal candidates for use as safe delivery vectors expressing therapeutic recombinant proteins [5]. In this respect, *Lactococcus lactis* and *Lactobacillus plantarum* are probably the two most popular LAB utilized as potential food-grade vectors [6]. *L. lactis* has traditionally been used for the manufacture of Swiss type cheese and is a reliable host for the heterologous expression of recombinant proteins [7,8]. *L. plantarum* is present in many probiotic formulations worldwide and has also been genetically modified for the *in vivo* therapeutic delivery of proteins to mucosal surfaces [9].

Tuberculosis (TB) still remains as one of the most deadly diseases affecting humans and animals worldwide [10,11]. The attenuated *Mycobacterium bovis* strain Bacillus Calmette-Guérin (BCG) is currently the only available TB vaccine but protection is incomplete. Furthermore, the use of BCG in cattle to protect against bovine TB (bTB) confounds screening for infection by skin testing due to antigen cross-reactions with *M. bovis* natural infection. Therefore, there remains a need to develop novel approaches to tackle bTB. Recent studies have suggested that LAB such as *L. plantarum* and *L. lactis* could be used as safe vectors for the delivery of TB antigens to mucosal sites [12,13]. The *M. tuberculosis* antigens Ag85B and ESAT-6 have been successfully expressed on the surface of *L. plantarum*, inducing TB-specific immune responses in mice after nasal or oral immunization [12]. Similarly, *L. lactis* has been used as a cell factory for the production of bio-beads that display the mycobacterial antigens Ag85A and ESAT-6 on their surface [13], resulting in a significant decrease in the *M. bovis* counts present in the lungs and spleen of mice vaccinated with the Ag85A/ESAT-6 bio-beads alone or in combination with BCG.

In this study we have explored the ability of *L. lactis* and *L. plantarum* to serve as heterologous hosts for the secretion and functional

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expression of MPB70 and MPB80, two of the most intensely studied *M. bovis* antigens to date [14]. MP70 is a soluble, secreted protein cleaved by type I-signal peptidase that has widely been used as a diagnostic marker [15]. MPB83 is a glycosylated lipoprotein processed by type II signal peptidase II and located at the bacterial membrane, which confers stronger immunogenic properties when compared to MPB70 [16]. We therefore generated recombinant cells of *L. lactis* and *L. plantarum* using plasmids that contained hybrid genes encoding the antigens MPB70 and MPB80 fused to signal peptides that are specifically recognized by LAB. Only *L. lactis* proved to be an efficient heterologous host for the functional expression of both antigens. MPB70 was secreted using an enhanced version of the *L. lactis* signal peptide Usp45, while the signal peptide of the *L. plantarum* lipoprotein prsA allowed the expression of immunogenic MP83.

2. Material and methods

2.1. LAB strains, competent cells and plasmids

The two LAB strains *Lactococcus lactis* NZ9000 and *Lactobacillus plantarum* WCFS1 were selected as the hosts for the heterologous expression of the *M. bovis* antigens MPB70 and MPB83. *L. lactis* NZ9000 is a derivative of the reference strain *L. lactis* MG1363 that carries the genes *nisR* and *nisK* integrated in its chromosomal DNA to enable heterologous protein expression with the nisin-controlled gene expression system (NICE) [17,18]. *L. plantarum* WCFS1 is a reference strain used as a model for probiotic studies [19]. Both LAB show high transformation efficiency and reliability for the expression of proteins under the control of LAB strong promoters and/or the NICE system [8,20,21]. NZ9000 and WCFS1 were grown in M17 medium (Oxoid) supplemented with 0.5% (wt/vol) glucose (GM17) at 30 °C, and their corresponding electrocompetent cells were generated as previously described [22–24]. The plasmid selected for the heterologous expression of MPB70 and MPB83 was pNZ8048c. This plasmid harbours a chloramphenicol selection marker, replicates efficiently in LAB and allows for high protein expression under the control of the NICE dependent promoter P_{nisA} [18,25]. Plasmid isolation from either *L. lactis* or *L. plantarum* was carried out using the EZNA®Plasmid Isolation kit (Omega Biotech) but with the cells previously suspended in lysis buffer consisting of: GTE solution (glucose 50 mM, Tris-HCL 25 mM pH8.0, EDTA 10 mM) with lysozyme (20 mg ml^{-1}) (Sigma) for *L. lactis*; TES (50 mM Tris pH7, 20 mM EDTA, 0.2 mM sucrose with lysozyme (10 mg ml^{-1}) and mutanolysin 70U.ml^{-1} for *L. plantarum*; and incubated at 37 °C for 15–60 min, before following the manufacturer's instructions.

2.2. DNA synthesis for the secretion of MPB70 in L. lactis and L. plantarum

Two DNA fragments encoding for the secreted form of MPB70 fused to specific signal sequences for *L. lactis* and *L. plantarum* were synthesized for optimal codon usage in LAB [26]: Usp45TM8:MPB70 and 3050:MPB70, respectively (Fig. 1A, Fig. S1-2). Usp45TM8 is a derivative of the *L. lactis* signal peptide Usp45 that allows for a much higher secretion efficiency when compared to Usp45 due to a combination of amino acid changes and silent mutations [27]. An additional sequence encoding nine amino acids (LEISSTCDA) was also included between the sequences for Usp45TM8 and MPB70 to enhance secretion in *L. lactis* [28]. 3050 is a signal peptide derived from the protein Lp_3050 of *L. plantarum* that generally results in higher levels of secreted proteins by comparison with other *L. plantarum* signal peptides [29]. Both signal peptides were designed to be easily exchangeable using the restriction enzymes *Pst*I and *Xba*I. Furthermore, the two synthesized DNA fragments contained other genetic elements including nucleotide sequences for the recognition of the restriction enzymes *Sac*I and *Hind*III to clone into pNZ8048c under the control of P_{nisA}; and optimal ribosomal binding site (RBS) for LAB; and a 6x-His Tag on the C terminus, to enable detection by Western blot, which was inserted using site-directed mutagenesis as indicated below.

2.3. DNA synthesis for the functional expression of MPB83 in L. lactis and L. plantarum

Two DNA fragments encoding for the functional form of MPB83 fused to specific lipoprotein signal sequences for *L. lactis* and *L. plantarum* were synthesized for optimal codon usage in LAB [26]: L88446:MPB83 and 1452:MPB83, respectively (Fig. 2A, Fig S3-4). L88446 and 1452 refer to specific anchors of lipoproteins OppA of *L. lactis* [30] and PrsA of *L. plantarum* [31], respectively. Both anchors contain a signal peptide for secretion followed by a lipobox sequence that allow for efficient protein display on the bacterial surface. As indicated above, the two synthesized DNA fragments carried other genetic elements including an optimal RBS for LAB, and nucleotide sequences for the recognition of the restriction enzymes *Sac*I and *Hind*III to facilitate insertion into pNZ8048c.

2.4. Expression of MPB70 and MPB83 in L. lactis and L. plantarum

The four synthesized DNA fragments; two for each of MPB70 and MPB83, as described above, were provided by Eurofins Genomics (Ebersberg, Germany) in a replicative *E. coli* pEX standard vector. Each fragment was inserted into pNZ8048c using the restriction enzymes *Sac*I and *Hind*III and the resulting ligation mixtures introduced into competent cells of *L. lactis* NZ9000 or *L. plantarum* WCFS1 with a BTX ECM® 399 Electroporator as previously reported [23]. All DNA-cloning enzymes were obtained from Thermo Scientific (Fast Digest and Ligase) and used as recommended by the supplier. Supernatants and pellets from cultures of the resulting recombinant strains were collected to evaluate MPB70/83 expression. The pNZ8048c recombinant strains were propagated until they reached an $OD_{600\ nm}$ of 0.5 (exponential phase) to activate the promoter P_{nisA} with a 4×10^{-3}-fold diluted supernatant of *L. lactis* NZ9700 (NisA producer), as previously described [8]. The cultures were further incubated for 3 h until reaching an OD_{600} of 1.0 prior to harvesting. To enable P_{nisA} activation in *L. plantarum* WCFS1, cells were transformed simultaneously with the erythromycin resistance vector pNZ9530 [18] carrying the genes *nisK* and *nisR*. The antibiotics chloramphenicol and erythromycin were provided by Apollo Scientific (UK) and used at concentrations of 5 µg.ml^{-1}.

2.5. Protein extraction from recombinant strains of L. lactis and L. plantarum

50 mL of recombinant cultures were placed on ice for 30 min prior to centrifugation at 3,000 g for 10 min at 4 °C. The supernatants were collected as the extracellular protein (EP) fraction, and the pellets, following a washing step in ice cold PBS, werere-suspended in 500 µL of ice-cold lysis buffer (70 mM Hepes pH8, 20 mM Imidazole, 650 mM NaCL) with 0.05% mercaptoethanol (Sigma). The pellet suspensions were then subjected to mechanical lysis by sonication using a probe (Soniprep 150 MSE) for 20s (5x), followed by a centrifugation at maximum speed for 10 min at 4 °C. The resulting supernatants were collected as the cytoplasmic protein (CP) fraction, and the pellets re-suspended in 300 µl of lysis buffer from which supernatants were obtained as the membrane protein (MP) fraction after sonication and centrifugation as described before. The three collected fractions (EP, CP and MP) were stored at −20 °C until further use.

2.6. Acetone precipitation for protein concentration

Filter sterilized supernatants containing the EP fraction with protease inhibitors (PMSF 1 mM final concentration) were concentrated using acetone precipitation. Four volumes of an ice cold (20 °C) solution of acetone was added to the supernatants, mixed vigorously with a vortex and placed at −20 °C overnight. Pellets from the samples were

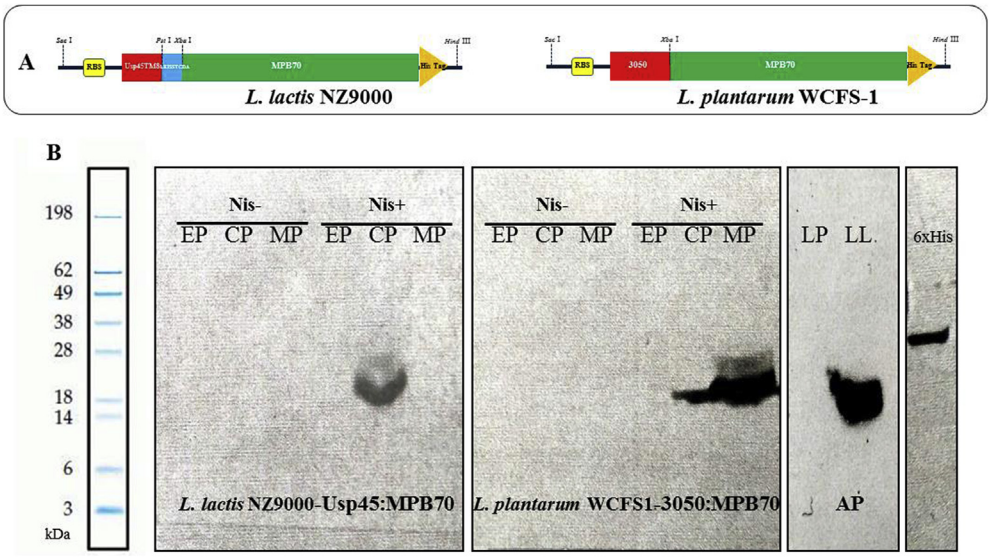

Fig. 1. (A) A diagram representing the two synthesized nucleotide sequences that were cloned in the nisin inducible vector pNZ8048e to allow the secretion of mature MPB70 in *L. lactis* NZ9000 and *L. plantarum* WCFS1. Both constructs contain sites for the recognition of restriction enzymes (dotted lines); ribosomal binding (yellow), signal peptide (red), MPB70 as secreted in *M. bovis* (green) and a 6x His Tag (orange). The *L. lactis* Usp45TM8 signal peptide is followed by the nine amino acid residue LEISSTCDA, while the *L. plantarum* 3050 signal peptide is fused directly to MPB70. **(B)** SDS-PAGE and Immunoblotting of samples containing extracellular protein (EP), cytoplasmic protein (CP) and membrane protein (MP) from recombinant cultures of *L. lactis* NZ9000-Usp45:MPB70 and *L. plantarum* WCFS1-3050:MPB70. The cultures were propagated in the presence (+) or absence (−) of nisin (Nis) and concentrated EP fractions from both *L. plantarum* (LP) and *L. lactis* (LL) were further tested following acetone precipitation (AP). Mouse anti-6x-His Tag, monoclonal IgGs were used as the primary antibodies and a cell lysate from *E. coli* cultures expressing a 30 KDa-His tag protein was the positive controls (6xHis). Usp45:MPB70 and 3050:MPB70 have a theoretical molecular weight of 21.4 and 21.5 KDa when translated in the cytoplasm, while their secreted versions weigh 18.6 and 17.37 Kda, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

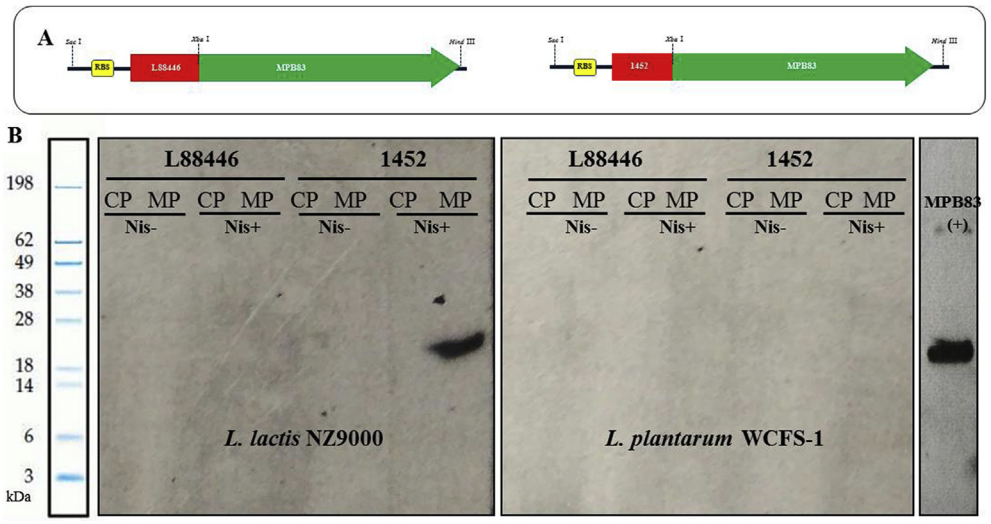

Fig. 2. (A) A diagram representing the two synthesized nucleotide sequences that were cloned in the nisin inducible vector pNZ8048e to allow the functional expression of mature MPB83 in *L. lactis* NZ9000 and *L. plantarum* WCFS1. Both constructs contain sites for the recognition of restriction enzymes (dotted lines); ribosomal binding (yellow), a signal peptide with a lipbox motif on the *C*-terminus (red) and the mature MPB83 with their corresponding lipbox on the *N*-terminus (green). L88446 and 1452 were selected as the signal peptides for the expression of MPB83 in both *L. lactis* and *L. plantarum*. **(B)** SDS-PAGE and immunoblotting of samples containing cytoplasmic protein (CP) and membrane protein (MP) from MPB83 recombinant cultures of *L. lactis* NZ9000 and *L. plantarum* WCFS-1. The cultures were propagated in the presence (+) or absence (−) of nisin (Nis). *Anti*-MPB83 monoclonal IgGs and pure recombinant MP83 were used as the primary antibodies and the positive control (+), respectively. L88446:MPB83 and 1452:MPB83 have a theoretical molecular weight of 22.6 and 22.7 Kda, when translated in the cytoplasm, while their processed lipoprotein versions weigh 20.5 KDa. When glycosylated the molecular weights raise up to approximately 25.5 KDa and 23.5 KDa for each of the versions. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

then obtained following centrifugation at maximum speed for 10 min at 4 °C and two washing steps with acetone:water solution (−20°c) at a ratio of 4:1. The pellets were air dried for 10–15 min and resuspended in sample buffer for SDS-PAGE and immunoblotting analysis.

2.7. MPB70 His-tag site-directed insertion and purification

In order to facilitate MPB70 detection in the fractions (EP, CP and MP) collected from the recombinant cells of *L. lactis* and *L. plantarum*, a polyhistidine tag (6x-His tag) was previously inserted at the end of the *C*-terminal region of MPB70 in the *E. coli* vector pEX using the Q5 Site-Directed Mutagenesis Kit (New England Biolabs). The His-tag insertion was conducted as indicated in the manufacturer's instructions using the forward primer MPB70SDM-Fwd (CATTAATAGAAGCTTGCTCGCTTGG ATCCGAAT) and the reverse primers MPB70SDM-Rev-lactis (GTGATG GTGATGGTGAGCTGGTGGCATAAGAAC) and MPB70SDM-Rev-plantarum (GTGATGGTGATGGTGAGCTGGTGGCATTAAAAC) for the pEX vectors carrying Usp45TM8:MPB70 and SP3050:MPB70, respectively. The pEX vectors were then digested with *Hind*III and *Sac*I and following gel extraction the fragments were cloned into a similarly digested pNZ8048 vector. The resulting vector was transformed into competent cells and the colonies screened by sequencing to confirm that the correct mutagenesis had occurred. Subsequent isolation of MPB70 was carried out using His Spin Trap GE Healthcare columns, following concentration in a centrifugal concentrator at 2,000 g for 10 min. A volume of 600 µL of each supernatant was put into the columns, previously equilibrated with binding buffer (70 mM Hepes pH 8, 20 mM Imidazole, 500 mM NaCl and 0.1 mM B-mercaptoethanol). All the columns were centrifuged at 100 g for 30s, and washed with binding buffer three times. Proteins were then eluted with 200 µl of elution buffer (60 mM Hepes pH 8.0, 400 mM Imidazole, 150 mM NaCl, 3% Glycerol) following centrifugation at 100*g* for 30s.

2.8. SDS-PAGE and immunoblotting

A volume of 40 µl containing the protein sample (EF, CF and NF) was prepared using Nupage® lithium dodecyl sulfate sample buffer (LDS, pH 8.4) and Nupage® sample reducing agent with dithiothreitol at 500 mM (Thermofisher Scientific). The 40 µl-samples were heated at 70 °C for 10 min, loaded onto a 4–12% polyacrylamide gel (Novex Bolt Bis-Tris) and run at 200 V for 22 min in 2-(*N*-morpholino) ethanesulfonic acid (MES) buffer in a Run Bolt Mini gel tank standard. Novex See Blue Plus®2 was used as the protein marker. The proteins were then transferred onto a PVDF membrane (Amersham Biosciences) by a semi dry transfer cell (Bio-Rad *Trans*-Blot SD) in a western tank buffer (0.303% Tris HCl, 1.44% Glycine, 20% Methanol) and run for 30 min at 22 V. The PVDF membrane was previously dipped in absolute methanol to rehydrate. The correct transfer of proteins on to the membrane was visualised by Ponceau red stain for 5 min. Once the proteins were transferred, the membrane was blocked with PBS containing 5% milk powder and 0.1% Tween 80 on a rotating platform for 1 h, followed by an incubation step at 37 for 1 h with primary antibodies at a concentration of 1:500 in the blocking solution. The primary antibodies used to detect the His-tagged MBP70 and the recombinant glycosylated MP83 were provided by Invitrogen (Mouse anti-6x-His Tag IgG, monoclonal antibody) and Lionex GmbH (Mouse *anti*-MPB83 IgG, monoclonal antibody), respectively. We then performed a series of washes with PBS-0.1% Tween 80 in order to incubate the membrane for a further hour with the secondary antibody at a concentration of 1:10,000 in blocking solution. HRP-Rabbit anti-Mouse polyclonal antibodies from Invitrogen were used as secondary antibodies. The membrane was finally washed three times as previously described and incubated with Horseradish Peroxidase (HRP) (West Pico) in a sufficient volume (0.1 mL of the working solution per cm^2 of membrane) for 5 min. The membrane was protected in cling film and exposed to lumifilm chemiluminescent detection film (Roche) for a period of time ranging from 5 min to overnight in order to gain the best exposure. The film was developed in a B&W Amfix TM manual X-ray developer for 1 min, washed in water and then fixed in B&W Amfix TM film & paper fixer for 1 min. The blot image was captured and visualized using Image J software. For the Western blotting we included a cell lysate from an *E. coli* culture expressing a 30 KDa-His tag protein and purified MPB83 recombinant protein at 5 $\mu g.ml^{-1}$ (Lionex GmbH) as positive controls.

THP-1 cell cultures. The THP-1 Lucia™ NF-κB monocyte cell line (Invivogen) was used to monitor the NF-κB signal transduction pathway in macrophages when exposed to cells obtained from the MPB83-expressing *L. lactis* recombinants. These monocytes secrete *Gaussia* luciferase under control of the NF-κB promoter and are grown in Roswell Park Memorial Institute (RPMI) 1640 medium (Life Technologies) supplemented with 15% foetal bovine serum (FCS, Seralab) and 1% Penicillin/Streptomycin (Pen/Strep, Life Technologies) at 37 °C in an atmosphere of 5% CO_2. In order to differentiate the monocytes into macrophages we used Thermo Scientific Nunc MicroWell™ 96-well plates to seed 5×10^4 cells per well in RPMI supplemented with phorbol 12-myristate 13-acetate (PMA) at 20 ng ml^{-1}. PMA was provided by Santa Cruz Biotechnology and dissolved in DMSO at 10 mg ml^{-1}. After 48 h, the medium was replaced with RPMI containing 2% FCS and 1% Pen/Strep and supplemented with inactivated *L. lactis* cells.

Luciferase measurements from THP-1 cells exposed to inactivated *L. lactis* cells. PMA-differentiated THP-1 cells were exposed to UV-inactivated *L. lactis* pellets that were resuspended in RPMI containing 2% FCS and 1% Pen/Strep. The *L. lactis* cells were inactivated using UV radiation as described previously [32] in order to visualise the immunogenic effect of intact membrane components. After 12 h of exposure, supernatants were transferred to white-bottom 96-well plates and luciferase activity was measured in the presence of 2 µg/mL of coelenterazine (NanoLigh Technology) using a Clariostar plate reader (BMG Biotech). MPB83 at 0.5 µg μl^{-1} and inactivated cells obtained from cultures of non-induced *L. lactis* recombinants and NZ9000 were used as controls. NF-κB activation was calculated as a fold increase over the measurements recorded for unchallenged macrophages.

3. Results

3.1. Secretion of MPB70 in L. lactis and L. plantarum

In order to determine whether LAB are capable of secreting the *M. bovis* antigen MPB70 we generated two recombinant strains: *L. lactis* NZ9000-Usp45TM8:MPB70 and *L. plantarum* WCFS1-3050:MPB70 (Fig. 1A). Both recombinants were created by transformation with a pNZ8048e vector that carries a gene encoding the secreted version of MPB70 fused to the signal peptide Usp45TM8 (or 3050) under the control of the NICE system. We also incorporated a 6x His Tag on the *C*-terminal region of MBP70 to facilitate protein purification. Recombinant cells were grown in the presence or absence of nisin to collect samples that were fractionated as extracellular, cytoplasmic or membrane protein for further analysis with SDS-PAGE and immunoblotting. As illustrated in Fig. 1B, MPB70 was detected in the cytoplasmic fraction of both recombinants following the incorporation of nisin. Under the same experimental conditions, the *L. plantarum* recombinant was also found to be able to express high levels of MPB70 in the membrane fraction. Following protein concentration with acetone precipitation, we detected MB70 in the extracellular protein fraction but only in samples derived from the supernatants of the *L. lactis* recombinant.

3.2. Expression of MPB83 in L. lactis and L. plantarum

The capability of LAB to produce MPB83 as a lipoprotein was tested using four recombinant strains: *L. lactis* NZ9000-L88446:MPB83, *L. lactis* NZ9000-1452:MPB83, *L. plantarum* WCFS1-L88446:MPB83 and *L. plantarum* WCFS1-1452:MPB83 (Fig. 2A). The four recombinants contain the nisin-inducible vector pNZ8048 with a gene that codes for the signal peptide L888446 (or 1452) followed by a mature version of MPB83. The gene was designed to ensure the expression of lipobox motifs at the *C*-terminus of each signal peptide (LSAC in L888446 and LAGC in 1452) and at the *N*-terminus of MPB83 (LAGC). Lipoprotein precursors are normally produced with a signal peptide that contains a consensus lipobox motif (LxxC) in its carboxyl region. The cysteine residue of this motif is then targeted by a diacylglyceryl transferase to cause a lipidation that serves not only as an anchor between the lipoprotein and the membrane but also as a recognition site for signal peptidases, which leave the lipobox cysteine as the new amino-terminal residue of the mature lipoprotein [33]. Therefore, our hybrid gene design offers two lipidation possibilities; either following the pathway natural to LAB or to mycobacteria. As indicated in Fig. 2B we only detected MPB83 in the membrane fraction sample obtained from the *L. lactis* recombinant 1452:MPB83.

NF-κB response in macrophages exposed to inactivated cells of 1452:MPB83 *L. lactis* recombinant. To study the immunogenic effect of MPB83 we used THP-1 macrophages carrying a NF-κB luciferase reporter as a model system. We assessed NF-κB responses at 100 inactivated bacterial cells per macrophage, a dose that we had previously observed to trigger NF-κB activation by inactivated LAB [34]. Exposure to cells obtained from *L. lactis* NZ9000-1452:MPB83 cultures that were grown in the presence of nisin significantly enhanced NF-κB activation in comparison to cells derived from the same cultures but propagated in the absence of nisin (Fig. 3A). We also observed that the combination of inactivated NZ9000 cells and MPB83 at 0.5 µg/mL resulted in a significant increase in the NF-κB response when compared to THP-1 macrophages only exposed to NZ9000 cells or MPB83 alone (Fig. 3B). No immunogenicity studies were carried out with the MPB70-expressing recombinants due to the lack of response of the THP-1 Lucia™ NF-κB macrophages to this antigen at a concentration as high as 0.5 µg/mL.

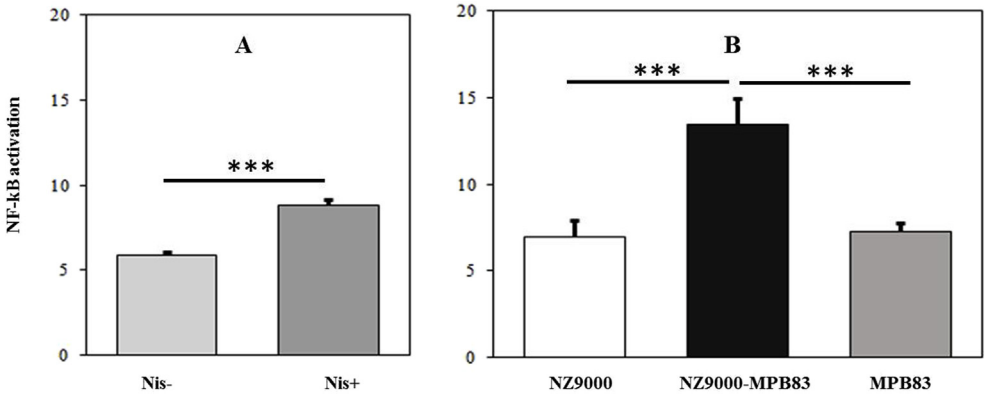

Fig. 3. NF-kB activation in PMA-differentiated THP-1 macrophages exposed to **(A)** inactivated cells of 1452-MPB83 recombinant *L. lactis* NZ9000 cultures that were propagated in the absence (−) or presence (+) of nisin (Nis); and **(B)** inactivated cells of *L. lactis* NZ9000 (white), MPB83 at 0.5 μg/mL (grey) and a combination of NZ9000 inactivated cells and MPB83 at 0.5 μg/mL (black). Data represent three biological replicates and the comparative statistical analysis was carried out using the Student *t*-test for panel A and ANOVA with post-hoc tests for panel B (***$p < 0.005$).

4. Discussion

Heterologous expression is a common strategy used extensively for the functional production of mycobacterial antigens that exhibit a high degree of specificity and immunogenicity [35,36]. This strategy is crucial for the improvement of diagnostic assays and vaccines against TB; and has been reported to be relatively efficient by following optimised codon usage in *E. coli* [37] or by exploiting *M. smegmatis* as a fast-growing mycobacteria species that facilitates natural antigen processing and secretion [38,39]. Although laboratory strains of *E. coli* and *M. smegmatis* are non-pathogenic, they do not enjoy a "generally recognized as safe" (GRAS) status, which is a desirable feature of an antigen-expression vehicle, especially one that might even be given as a vaccine to animals or humans. In this respect, LAB offer the possibility to developing GRAS-based, heterologous expression systems to efficiently produce *M. tuberculosis* antigens [12,13]. Furthermore, due to their probiotic properties, recombinant LAB are also capable of eliciting specific systemic and mucosal immune responses against selected antigens [6]. In this study we report for the first time, the heterologous production of the *M. bovis* antigens MPB70 and MPB83 in LAB. In order to attain high protein expression we cloned *mpb70* and *mpb83* downstream of the NICE dependent promoter P_{nisA} on a plasmid also carrying a chloramphenicol selection marker. Future application of recombinant LAB for administration to animals or humans would require construction of a stable recombinant strain devoid of any antibiotic resistance genes.

The *L. lactis* recombinants that we generated using the Usp45 signal peptide secreted the mature version of MPB70. The Lysine residue that we incorporated immediately after the peptidase recognition site may be essential for MPB70 secretion as previously reported with other recombinant proteins [28]. For the secretion of MPB70 in *L. plantarum*, we employed an identical strategy but using the signal peptide 3050 and the incorporation of an Serine residue along with the peptidase recognition site to facilitate extracellular production, as it occurs naturally in *L. plantarum* [29]. However, we could not detect MPB70 in the supernatants obtained from cultures of the *L. plantarum* recombinants. The fact that the other fractions – cytoplasmic and membrane-showed high levels of expression of the MPB70 hybrid protein suggest that peptidases do not efficiently recognize 3050 in our recombinant cells. However, considering that 3050 is probably the most efficient signal peptide for the secretion of recombinant proteins in *L. plantarum* ([40], we are more inclined to propose other reasons for the absence of extracellular MPB70, including proteolytic activity. Wild-type LAB strains such as *L. plantarum* WCFS1 produce a significant number of proteases [41] that might be responsible for a reduced antigen-epitope recognition on the immunoblotting assay. The reason why MPB70 is not detected in the membrane fraction of the *L. lactis* recombinant cells could also due to this reduction in antibody recognition, but probably due to other circumstances such as protein folding or interaction with other membrane compounds. Furthermore, the use of antibodies directed to His-tag and not to MPB70 is a limitation of our study design that would need to be addressed using *anti*-MPB70 antibodies.

Our Western blot analysis has shown that MPB83 is produced as a membrane protein in *L. lactis* recombinant cells carrying the gene encoding the mature version of MPB83 fused to the lipoprotein signal peptide 1452 of *L. plantarum*. By contrast, L88446, a lipoprotein signal peptide of *L. lactis*, was unable to produce MP83 at the membrane level. Our results suggest that the lipobox motifs located at the *C*-terminal region of the selected signal peptides are differently recognized by diacylglyceryl transferases. LAGC, the lipobox motif of the 1452:MP83 protein, could be recognized more efficiently than the lipobox motif present in L8846:MP83 (LSAC), resulting in a better peptidase recognition and membrane anchoring. Furthermore, it is likely that the lipobox motif that is located at the *N*-terminus of the mature MPB83 played no role in the recognition of such transferases. Surprisingly, the transformation of *L. plantarum* with the 1452:MP83 plasmid showed no MPB83 production in the membrane fraction, which could be due to the anchoring method selected in this study. Recently, successful surface display of a *M. tuberculosis* fusion antigen using two different anchors: an *N*-terminal lipoprotein anchor directing the protein to the cell membrane and a *C*-terminal covalent cell wall anchor, has been reported [12]. However, the use of multiple anchoring strategies has shown that antigens may distribute in *L. plantarum* as cell membrane-anchored, cell wall-anchored, and secreted, in nearly equal amounts but also randomly [42], which suggest that anchors may not be that essential for cell membrane/wall localization.

None of the cytoplasmic fractions obtained from recombinant cells of *L. lactis* and *L. plantarum* contained detectable amounts of MPB83. In *M. bovis* and *M. tuberculosis*, glycosylated MPB83 is located at the bacterial surface, possibly with the lipid tail coupled to the *N*-terminal cysteine embedded in the outer membrane [16]. The non-glycosylated form of MPB83 is usually present in the cytoplasm. The monoclonal antibody used in this work binds to glycosylated MP83 only and could explain why we could not detect the antigen in the cytoplasm of the recombinant LAB.

In this study we also observed that inactivated cells obtained from cultures of the *L. lactis* 1452:MPB83 recombinant induced NF-κB activation in the human THP-1 macrophage cell line. NF-κB induction was significantly higher in the presence of nisin, suggesting that MPB83 is being functionally expressed in the membrane of the recombinant cells. The combination of inactivated cells of *L. lactis* and purified MPB83 resulted in an enhanced NF-κB response compared with incubation of THP-1 cells with either MPB83 or *L. lactis* alone, suggesting a synergistic effect. On the basis of other work [43,44], it is possible this is synergistic effect is mediated through TLR-2. Both LAB and MPB83 normally activate macrophages in a TLR-2-dependent manner.

In conclusion, our study has shown that LAB, and in particular *L. lactis*, is a reliable host for the secretion and functional expression of MPB70 and MPB80, two *M. bovis* antigens that are of interest due to

their diagnostics and immunogenic properties. Governments around the globe have made a significant effort and investment in reducing the incidence of bTB. However, figures show no progress at all, with estimated costs of around £100 million per year in the UK alone [45]. The use of recombinant LAB as a food-grade vector for the production and delivery of bTB antigens such as MPB83 for further application as novel vaccine biomaterials and/or mucosal adjuvants is an approach worthy of further exploration. The MPB70 hybrid construction could also serve as a model for the secretion and purification of other bTB antigens that are of interest as diagnostic reagents.

Conflicts of interest

No conflict of interest declared.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.tube.2019.05.007.

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