Engineering Lactobacillus rhamnosus GG and GR-1 to express HIV-inhibiting griffithsin

Highlights

- *Lactobacillus rhamnosus* GG and GR-1 can be used as delivery vehicles for the expression of recombinant anti-HIV proteins
- Expression of the anti-HIV lectin griffithsin was achieved under the control of the NICE system
- This recombinant GRFT expressed in *L. rhamnosus* strains showed strong anti-HIV activity against T-tropical (X4) HIV-1 strain NL4.3 and M-tropic (R5) HIV-1 BaL strain
- Actinohivin, the other well-known anti-HIV lectin, seemed toxic for the *Lactobacillus* strains when expressed in the cells

Engineering *Lactobacillus rhamnosus* GG and GR-1 to express HIV-inhibiting griffithsin

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Running title: Heterologous expression of anti-HIV lectins in *L. rhamnosus*

Abstract

Probiotic bacteria are being explored for the *in situ* delivery of various therapeutic agents. In this study, we aimed to express two HIV-inhibiting lectins, actinohivin (AH) and griffithsin (GRFT), in the probiotic strains *L. rhamnosus* GG and *L. rhamnosus* GR-1 for gastrointestinal and vaginal mucosal delivery, respectively. Hereto, constructs were generated for the intracellular and extracellular production of AH and GRFT under the control of the promoter of their Major Secreted Protein Msp1. Also, intracellular expression of GRFT was investigated under the control of the *nisA* promoter from the inducible NICE system. For the extracellular localization, the signal leader peptide of Msp1/p75 from *L. rhamnosus* GG was translationally fused with the genes encoding AH and GRFT. Construction of recombinant strains expressing the AH monomer and dimer was unsuccessful, probably due to the intracellular toxicity of AH for the lactobacilli. On the other hand, recombinant strains for intra- and extracellular production of GRFT by *L. rhamnosus* GG and GR-1 were successfully constructed. The highest expression levels of recombinant GRFT were observed for the constructs under the control of the inducible *nisA* promoter and we demonstrated anti-HIV activity against an M-tropic and a T-tropic HIV-1 strain. We can conclude that recombinant *Lactobacillus* expressing anti-HIV lectins could contribute to the development of enhanced probiotic strains that are able to inhibit HIV transmission and subsequent replication, although further research and development are required.

Keywords: probiotics, lectins, carbohydrate-binding agents, actinohivin, griffithsin, HIV, *Lactobacillus rhamnosus*.

1. Introduction

With human immunodeficiency virus (HIV) infections being one of the top infectious diseases worldwide, the interest in carbohydrate-binding agents (CBAs) as therapeutic agents that can block HIV is growing [1]. Two promising peptidic CBAs, with documented activity against HIV and so far unknown side-effects for human host cells, are actinohivin (AH) and griffithsin (GRFT) [2, 3]. AH is a 12.5 kDa lectin isolated from the actinomycete *Longisporum albida* [4, 5]. AH consists of 114 amino acid residues, divided into three segments, forming three sugar-binding pockets to accommodate Man-α(1-2)-Man residues of N-linked glycans present on the surface of the HIV envelope glycoprotein gp120, therefore inhibiting HIV infection [3, 5, 6]. AH has been shown to prevent T-cell and macrophage (M)-tropic syncytium formation by HIV [4], has no mitogenic activity and does not induce secretion of inflammatory cytokines or chemokines in peripheral blood cells [7]. GRFT, isolated from the red alga *Griffithsia* sp., is a 12.7 kDa lectin consisting of 121 amino acids [2, 8]. The GRFT molecules form a domain-swapped dimer, providing six independent binding sites for monosaccharides [9]. GRFT binds strongly to the HIV-envelope glycoproteins gp120, gp160, and gp41, and at the same time shows an outstanding safety profile towards human epithelial and immune cells [10]. GRFT has been shown to inhibit HIV-induced cell killing in a concentration-dependent manner and to be active against T- and M-tropic strains of HIV-1 [2].

On the other hand, lactobacilli that are present on (vaginal) mucosal surfaces are increasingly considered as natural factors that can promote the host defense against HIV[11]. Furthermore, they can also be applied as probiotics to improve human lives or to prevent HIV infections. Probiotics are known as “live microorganism that, when administrated in adequate amounts,

confer health benefits in the host" [12]. For example, the well-known and widely used urogenital probiotic strain *L. rhamnosus* GR-1 can colonize the vaginal niche, as well as inhibit the growth and adhesion of a number of bacterial and viral pathogens [13, 14]. Furthermore, oral administration of *Lactobacillus rhamnosus* GR-1 and *Lactobacillus reuteri* RC-14 to HIV/AIDS patients has been associated with the resolution of diarrhoea, nausea, and flatulence [15], increased $CD4^+$ T-cell counts and reported longer working days [16, 17]. Therefore, *L. rhamnosus* GR-1 can be considered as a good probiotic able to restore health conditions both in the gastrointestinal tract and in the vaginal niche. Since probiotic bacteria are able to exclude pathogens in the human host and to improve human health, they also have a great potential to serve as a delivery vehicle for microbicides, such as anti-HIV molecules. In this way, the probiotic strains can be used not only as a delivery system but also as adjuvants stimulating the immune function and as agents that reduce symptoms often associated with HIV infections (e.g., gastrointestinal problems).

In this study, the well-documented probiotic strains *L. rhamnosus* GG and *L. rhamnosus* GR-1 were genetically engineered for mucosal oral and/or vaginal delivery of AH and GRFT as anti-HIV CBAs.

2. Material and Methods

2.1.Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1 and in more details in Table S1 and S2 . *L. rhamnosus* GG and GR-1 wild-type, as well as the corresponding recombinant strains, were routinely grown under anaerobic conditions in MRS medium (Difco™ 288130, Belgium) [18] at 37° C. *Escherichia coli* strains were grown in LB medium with aeration at 37°C. If required, antibiotics were used at following concentrations: 100 µg/ml ampicillin and 5 µg/ml (*Lactobacillus*) or 130 µg/ml (*E. coli*) erythromycin. Production of recombinant proteins by *L. rhamnosus* strains was induced with nisin as previously described [19].

Routine molecular biology techniques were performed [20]. PCR primers used in this study were purchased from Integrated DNA Technologies (Belgium) (Table S3). Enzymes for molecular biology were purchased from New England Biolabs. Plasmid DNA preparation from *E. coli* was performed using QIAGEN miniprep kits.

2.2.Construction of recombinant Lactobacillus strains for expression of AH

Intracellular expression of AH monomer (AHm) and AH dimer (AHd) was achieved under the control of the constitutive promoter of the Major secreted protein 1 (Msp1) of *L. rhamnosus* GG [21]. The genes were successfully cloned into pCMPG10750. One integrant that had the correct structure was selected and designated as pCMPG10754 for the AHm and pCMPG10755 for the AHd (Figure 1A) and electroporated into highly competent *L. rhamnosus* GG and GR-1 cells. Two colonies of *L. rhamnosus* GG, containing plasmids pCMPG10754 and pCMPG10755 were

designed as CMPG10761 and CMPG10762, respectively. For *L. rhamnosus* GR-1, also two colonies containing plasmids pCMPG10754 and pCMPG10755 were selected and named CMPG10763 and CMPG10764, respectively.

Extracellular expression was achieved by using the signal leader peptide (SLP) from the Msp1 of *L. rhamnosus* GG [21]. Hereafter AHm and AHd without N-terminal His_6-Tag were cloned into plasmid pCMPG10724. The plasmids were designated pCMPG10726 and pCMPG10738 for the AHm and AHd, respectively (Figure 1B). These plasmids were transformed into *L. rhamnosus* GG and GR-1. Also, the genome-integrating plasmid pCMPG10725 was included to achieve stable expression of AHm and AHd in *L. rhamnosus* GG and GR-1. One colony for AHm and AHd was chosen and designated as pCMPG10737 and pCMPG10739, respectively (Figure 1C). Subsequently, the corresponding plasmids were transformed into *L. rhamnosus* GG and *L. rhamnosus* GR-1.

2.3.Construction of recombinant Lactobacillus strains for expression of GRFT

Intracellular expression of GRFT was investigated under the control of *msp1* and *nisA* promoters. Hereafter the GRFT gene, without N-terminal His_6-Tag sequence, was cloned into plasmids pCMPG10750 and pCMPG10751 containing the *msp1* promoter, resulting in plasmids pCMPG10752 and pCMPG10753 (Figure 2A and B). pCMPG10752 was transformed to *L. rhamnosus* GG and GR-1 which resulted in recombinant strains CMPG10757 and CMPG10758, respectively. The electroporation of pCMPG10753 into *Lactobacillus* cells resulted in strains CMPG10759 and CMPG10760 for *L. rhamnosus* GG and GR-1, respectively. The GRFT gene was also cloned into pMEC45, containing the inducible *nisA* promotor, either with or without the

N-terminal His_6-Tag sequence resulting in plasmids pCMPG10776 and pCMPG10777, respectively (Figure 2C). Each plasmid was transformed into *L. rhamnosus* GG resulting in recombinant strains CMPG10778 and CMPG10780, containing pCMPG10776 and pCMPG10777, respectively. Electroporation was also performed for *L. rhamnosus* GR-1, resulting in CMPG1779 and CMPG10781 containing pCMPG10776 and pCMPG10777, respectively.

To obtain extracellular expression of GRFT, the *msp1* promoter and SLP of the *msp1* gene were used. First, the GRFT gene was amplified with an N-terminal His_6-Tag sequence, and cloned into plasmid pCMPG10724, resulting in plasmid pCMPG10729 (Figure 2D). Subsequently, pCMPG10729 was transferred to *L. rhamnosus* GG and *L. rhamnosus* GR-1. Correct strains were designated CMPG10733 and CMPG10734 for *L. rhamnosus* GG and GR-1, respectively. To achieve a stable expression, the GRFT gene was cloned into plasmid pCMPG10725. One construct was selected and designated as pCMPG10728 (Figure 2E). pCMPG10728 was transferred into *L. rhamnosus* GG and *L. rhamnosus* GR-1, resulting in recombinant strains CMPG10731 and CMPG10732, respectively. Finally, to achieve a better secretion of the recombinant GRFT, the N-terminal His_6-Tag was removed resulting in pCMPG10765 (Figure 2F), which was subsequently transformed into *L. rhamnosus* GG and GR-1. Colonies containing the pCMPG10765 were selected for future experiments and designated as CMPG10767 and CMPG10768 for *L. rhamnosus* GG and GR-1, respectively.

2.4. Protein purification

Protein purifications from recombinant and wildtype *L. rhamnosus* GG and GR-1 were performed as previously described with minor modifications [22, 23]. Briefly, secreted proteins were precipitated from the supernatant by incubation for one hour at 4°C in the presence of 70-85% ammonium sulfate (BCB-J0660V, Sigma, Belgium). After centrifugation for 30 min at 10,500 ×g, the pellet containing secreted proteins was resuspended in 1 ml PBS.

To purify non-covalently bound proteins from the cell wall, the pelleted cells harvested after centrifugation were washed twice by resuspending them in 25 ml and 15 ml PBS, respectively, and centrifuged for 20 min at 3,000 × g at 4°C. Subsequently, the pellets were resuspended in 1ml 10 mM Tris HCl, 1.5 M LiCl and incubated for one hour at 4°C. This solution was then centrifuged for 15 min at 5,000 × g. The obtained after centrifugation supernatant contained the cell wall non-covalently bound proteins.

To obtain the soluble cytosolic fraction of proteins, the pelleted cells harvested by centrifugation were washed twice with PBS, subsequently re-suspended in 2 ml of PBS containing 5 µl protease inhibitors cocktail (SRE0055, Sigma, Belgium), followed by two minutes sonication (8 sec on and 4 sec off with 10% amplitude) to disrupt the cells. After sonication, the cells were centrifuged for 30 min at 3,000 × g at 4°C. The collected supernatants contained soluble cytosolic proteins which were subject of SDS-PAGE and blotted

The proteins in each of the isolated fractions were separated by SDS-PAGE in NuPAge 12% BIS-Tris gels (NW00125, Invitrogen, Belgium), which was used to perform a Western Blot. Recombinant proteins were detected by using specific antibodies, i.e., anti-His_6 antibodies (11922416001, Roche, Belgium) at a concentration of 0.2 µg/ml, polyclonal anti-AH antibodies at a concentration of 0.3 µg/ml and polyclonal anti-GRFT antibodies diluted 1:10,000.

Subsequently, secondary anti-mouse (A3562, Sigma, Belgium) or anti-rabbit (A3687, Sigma, Belgium) antibodies conjugated with alkaline phosphatase at a dilution of 1:10,000 were used.

2.5. Anti-HIV replication assay

The firefly luciferase and *Escherichia coli* β-galactosidase expressing CD4+, CXCR4+, CCR5+ TZM-bl cells (50 µl; 2x105 cells/ml) were resuspended in cell culture medium supplemented with 15 µg/ml diethylaminoethyl-dextran (DEAE-Dextran; Sigma-Aldrich, Diegem, Belgium) and pre-incubated with 100 µl control compounds for 30 min. at 37°C in 96-well plates with cell culture medium. Recombinant griffithsin present in the cytosolic protein fraction of recombinant *Lactobacillus* strains was also added in its appropriate dilutions. As control compounds AMD3100 (CXCR4 inhibitor) and maraviroc (CCR5 inhibitor) and griffithsin were included. Then, the T-tropic (X4) HIV-1 strain NL4.3 or the M-tropic (R5) HIV-1 BaL was added (in 50 µl) according to the TCID50 ($TCID_{50}$ median tissue culture infective dose; that amount of a pathogenic agent that will produce pathological change in 50% of cell cultures inoculated) of the viral stock. Two days post-infection, viral replication is measured by luminescence. Steadylite plus reagent (Perkin Elmer, Zaventem, Belgium) was mixed with lyophilized substrate according to manufacturer's guidelines. Supernatant (120 µl) was removed and 75 µl Steadylite plus substrate solution was added to the 96-well plates. Next, the plates were incubated in dark for 10 min. in a closed plate shaker (PHMP, Grant, Shepreth, Cambridgeshire, UK). Finally, cell lysis was scored microscopically and 100 µl supernatant was transferred to white 96-well plates (Greiner Bio-One, Frickenhausen, Germany) to measure the relative luminescence units (RLUs) using the SpectraMax L microplate reader and Softmax Pro software (Molecular Devices, Sunnyvale, USA) with an integration time of 0.6 sec. and dark adapt of 5 min[24].

2.6 Statistical analysis

To determine significant differences between recombinant strains and wild type strains (negative controls) the unequal variance t-test and ordinary one-way ANOVA with Tukey's multiple comparison test was used. A value of $p \leq 0.05$ was considered to be statistically significant. All data were analysed using GraphPad Prism 7.

3. Results

3.1. AH cannot be expressed intracellularly and extracellularly by L. rhamnosus

Intracellular expression of AH monomer (AHm)and AH dimer (AHd) was investigated under the control of the *msp1* promoter. The successfully constructed recombinant *L. rhamnosus* GG and GR-1 strains were checked for production of AH intracellularly by Western Blot with monoclonal anti-His_6 antibodies and polyclonal anti-AH antibodies. Although at DNA level, the bacterial strains appeared to contain the constructs with the correct DNA sequence, no protein bands were detected at the expected size for all strains tested (data not shown).

For extracellular expression of AH, plasmids pCMPG10726 and pCMPG10737 were transformed into *L. rhamnosus* GG and GR-1 cells. However, no colonies were obtained after 12 independent electroporation rounds. The fact that no correct colonies were obtained, and that the transformation efficiency was significantly reduced, suggests a toxic effect of AH upon extracellular expression. Of note, transformed colonies were obtained in *L. rhamnosus* GG and GR-1 after transformation of pCMPG10738 and pCMPG10739 containing the AHd. However, sequence analysis of the bands revealed a deletion of one bp (the first G in the sequence GTG) at the beginning of the AHd gene, resulting in the insertion of a stop codon, further indicating the toxicity of AH. The same null mutation was observed already in *E. coli* while constructing plasmids pCMPG10738 and pCMPG10739 carrying the AHd. Therefore, the construction of pCMPG10738 and pCMPG10739 in *E. coli* was repeated five additional times, but the same null mutation was observed in all cases.

3.2. GRFT can be expressed intracellularly under the control of the inducible nisA promoter

Because AH turned out to be difficult to express in lactobacilli, we subsequently explored the possibility of heterologous expression of GRFT, which is also more active (see introduction). The GRFT gene was successfully cloned under the control of a constitutive promoter (*msp1)* and the inducible *nisA* promotor and transformed in *L. rhamnosus* GG and GR-1. However, expression of GRFT under the control of the *msp1* promoter did not result in distinct proteinbands by Western blot analysis with the polyclonal GRFT antibody of the cytosol fractions of CMPG10757, CMPG10758, CMPG10759 and CMPG10760 (Figure 3A and 3B). In contrast, strains carrying the GRFT gene with His_6-Tag downstream of the *nisA* promoter showed expression of GRFT. The highest intracellular expression was observed under the control of the *nisA* promoter for strains CMPG10778 (LGG-derived) and CMPG10779 (LGR-1-derived) (Figure 3C). No expression was detected for strains CMPG10780 and CMPG10781, expressing GRFT without N-terminal His_6 -Tag in *L. rhamnosus* GG and GR-1, respectively.

3.3. GRFT can be expressed extracellularly under the control of the msp1 promoter and the SLP

Subsequently, the *msp1* promoter and signal leader peptide of the Major Secreted protein of LGG and GR1 (Lebeer et al., 2012) were used to achieve extracellular secretion, hypothesizing that these genetic elements could also mediate high expression of GRFT. Based on the high expression of GRFT with N-terminal His_6-Tag under the control of the *nisA* promoter, we first amplified the GRFT gene with an N-terminal His_6-Tag, resulting into plasmid pCMPG10729. Stable expression of GRFT in *L. rhamnosus* GG and GR-1 was achieved by constructing pCMPG10728. To achieve a better secretion, the N-terminal His_6-Tag was removed resulting in pCMPG10765. All plasmids were successfully transferred into *L. rhamnosus* GG and GR-1.

The strains CMPG10733 and CMPG10734, carrying plasmid derivatives of the self-replicative pCMPG10724 vector, as well as strains CMPG10731 and CMPG10732 carrying plasmid derivatives of pCMPG10725 integrating into the chromosome of the *Lactobacillus,* were tested for secretion of GRFT by Western blot. No bands were observed for strains CMPG10733 (*L. rhamnosus* GG carrying GRFT with N-terminal His_6-Tag) in all protein fractions – cytosolic, cell wall and supernatant- tested (Figure 4A). For strain CMPG10734 (*L. rhamnosus* GR-1 carrying GRFT with N-terminal His_6-Tag), one protein band around 15 kDa (size cfr. GRFT with N-terminal His_6- Tag) was observed in the cell wall-protein fraction, but not in the cytosolic and supernatant fractions (Figure 4A), indicating secretion of the recombinant GRFT.

Two unique bands were detected in the cytosolic protein fractions of CMPG10731 and CMPG10732, *L. rhamnosus* GG and GR-1 (Figure 4B). One of the protein bands showed a size around ~18kDa, indicative of GRFT (12.6 kDa) with the SLP (3.8 kDa) of Msp1 and the His_6-Tag (1.5 kDa) (Figure 4B). The second protein band showed a size of ~15 kDa, indicative of GRFT (12.6 kDa) with the N-terminal His_6-Tag (1.5 kDa). Of note, in the cell wall and supernatant protein fraction, a band around 15 kDa (size cfr. GRFT with N-terminal His_6-Tag) could also be detected for CMPG10731 (*L. rhamnosus* GG carrying GRFT with N-terminal His_6-Tag) (Figure 4B), although the concentration was lower than in the cytosolic protein fraction. A relatively high concentration of the recombinant GRFT without N-terminal His_6-Tag (~16 kDa with SLP) was also observed in the soluble cytosolic fraction of strains CMPG10767 and CMPG10768 carrying the pEM40 derivate pCMPG10765 (Figure 4C). Also, two lighter bands were observed for both strains in the cell- wall fraction, indicating secretion of the recombinant

GRFT. The first protein band showed a size around ~16kDa, indicative of GRFT (12.6 kDa) with the SLP (around 3.8 kDa) of Msp1 protein (Figure 4C). The second protein band showed a size around ~13 kDa, indicative of GRFT (12.6 kDa). No visible bands were observed when testing the supernatant fraction of CMPG10767 and CMPG10768.

3.4. The anti-HIV activity of the recombinant GRFT from L. rhamnosus GG and GR-1

To investigate the anti-viral activity of the various recombinant GRFT constructs described above, protein fractions were isolated from the different recombinant *L. rhamnosus* strains and tested for inhibition of HIV infection and subsequent replication. Although Western blot analysis showed expression of recombinant GRFT, no specific antiviral activity was observed when testing the cytosolic and supernatant fraction of the recombinant strains designed to express GRFT extracellularly (strains, CMPG10734, CMPG10731, CMPG10732, CMPG10767, CMPG10768). In contrast, potent antiviral activity was observed for the recombinant GRFT expressed under the control of the *nisA* promoter (Table 2). Cytosolic protein fractions of strain CMPG10778 and CMPG10779 were able to inhibit T-tropic (X4) HIV-1 NL4.3 infection with an EC_{50} value of 1/1710 ($p=0.02$) and 1/3021 ($p=0.01$), respectively. Cytosolic protein fractions of strain CMPG10780 and CMPG10781 also showed weak, no-significant activity against HIV infection, although no clear bands were observed during the Western blot. Also, both CMPG10778, and CMPG10779, showed signifcant activity against the M-tropic (R5) HIV-1 BaL strain, with a dilution factor of 1/605 ($p=0.02$) and 1/1143 ($p=0.04$), respectively. No significant differences were observed between the activity of strains CMPG10778 and CMPG10779 against both T-tropic (X4) HIV-1 NL4.3 and M-tropic (R5) HIV-1 BaL strains.

4. Discussion

In this study, we investigated the heterologous expression of two HIV-inhibiting lectins, namely AH and GRFT by two well-known *Lactobacillus* probiotic strains *L. rhamnosus* GG and GR-1 [25, 26]. These two lectins, AH and GRFT, have a well-documented activity against HIV and unknown, if any, observed side-effects on the host cells [7, 27].

First, we optimized the genetic toolbox, including a selection of vectors with strong (endogenous) promoter for recombinant expression and constructs for different cellular localization of the recombinant proteins in *L. rhamnosus* GG and GR-1. Unfortunately, we were not able to obtain intracellular and extracellular expression of AH under the control of the *msp1* promoter. A possible explanation for the observed results might be that the AH is toxic during export out of the cell wall of *L. rhamnosus* GG and GR-1, possibly by binding to essential glycosylated cell wall molecules, such as peptidoglycan, exopolysaccharides or glycosylated proteins of the Sec pathway [28]. The toxicity of AH was further proposed by the occurrence of a nonsense mutation (stop codon) in the constructs pCMPG10738 and pCMPG10739 carrying the AH dimer upon transformation to *E. coli* TOP10 cells and *Lactobacillus*.. To the best of our knowledge, a toxic effect of AH against other bacterial or human epithelial cells has never been reported before and also AH did not show toxicity to a variety of lactobacilli when administered extracellularly in the growth medium [29].

Successful expression of intracellular and extracellular recombinant GRFT by lactobacilli was achieved here. Stable expression of recombinant GRFT under the control of the inducible *nisA* promoter was observed. The food-grade nisin-controlled gene expression system has been

previously used in *L. lactis* [30], *L. plantarum* [31], *L. rhamnosus* GG and GR-1 [19] for expression of various molecules. The extracellular expression of GRFT was investigated by using the promoter region and the SLP of the *msp1* gene. Only for the recombinant strain CMPG10731 (*L. rhamnosus* GG carrying GRFT with a His_6-Tag), a light band with correct size could be detected suggesting that GRFT is secreted although in very low concentrations. Of note, to improve the secretion of the recombinant GRFT, the His_6-Tag was removed in strains CMPG10767 and CMPG10768. Previous work in *L. plantarum* NCIMB8826 and *L. lactis* MG1363 has demonstrated that efficient protein translocation across the membrane requires an acidic N-terminus of the mature protein [32]. Therefore we hypothesized that direct fusion of GRFT with an acidic sequence of the mature Mps1 would enhance secretion efficiency of GRFT. However, Western blot analysis of the supernatant protein fractions did not show any enhanced secretion of GRFT. Since GRFT was not conclusively detected in the supernatant protein fraction, we investigated if it could be hampered by binding to cell wall glycan. Indeed, for recombinant CMPG10731, CMPG10734, CMPG10767 and CMPG10768 strains, bands corresponding to GRFT were detected in the cell wall fractions, suggesting possible trapping of the recombinant protein in the cell wall. Of note, high concentrations of GRFT were detected in the cytosolic fraction, highlighting the strong effect of the *msp1* promoter. The obtained results suggest that *L. rhamnosus* strains can synthesize GRFT, but not to secrete it out of the cells under the tested conditions. The lectin cyanovirin was already reported to be successfully expressed and secreted, after some optimizations, by *Lactobacillus jensenii* 1153 [32, 33]. Nevertheless, cyanovirin showed to have undesirable side-effects in host cells, by inducing the production of pro-inflammatory cytokines and chemokines, showing mitogenic activity and T cell proliferation [34], which makes it an unappropriated candidate for use in humans.

A critical validation step was to test whether our newly designed recombinant GRFT resembled the same HIV-inhibiting activity resembles the purified GRFT from *E.coli*. No activity was observed when testing the cytosolic fractions of the *Lactobacillus* strains producing recombinant GRFT for extracellular expression. This can be due to the presence of the SLP of Msp1, which might interfere with the activity of the recombinant GRFT or incorrect folding of the recombinant GRFT. Higher inhibition against both T-tropic and M-tropic HIV-1 strains was observed only when testing the strains producing recombinant GRFT under the control of the inducible *nisA* promoter. The observed inhibition was higher for the recombinant GRFT produced by the vaginal probiotic strain *L. rhamnosus* GR-1. Using probiotic *Lactobacillus* strains as a delivery system of anti-HIV molecules in the vagina is particularly interesting since the vaginal microbiota is mainly *Lactobacillus*-dominated and the vaginal epithelium is the main entry port for sexually transmitted HIV [11]. Nevertheless, the use of recombinant strains for delivery of therapeutic molecules must be seriously considered from biosafety perspectives and strictly regulated. For example, EFSA regulation claims that purity, stability, and potency of the gene-modify organisms (GMOs) have to be tested and well documented. Further, the presence of resistant cassettes and plasmids, virulence, pathogenicity, ability to colonize the host, full characterization of donor DNA, animal and human trials are mandatory when using GMOs. Therefore, future studies need to focus on optimization of the delivery systems by removing the antibiotic-resistant markers from the strains. Also, choosing appropriate promoters active only *in vivo* in the human gastrointestinal tract or the vaginal niche might contribute to a better delivery of the chosen molecules and strict control of the expression of recombinant proteins only in the human body.

In conclusion, the development of an effective HIV-1 microbicide is challenging. The new products must target conserved elements found in a genetically diverse group of viruses. The microbicides formulation should also provide sufficient mucosal levels over extended time periods. Administration in the vaginal or gastrointestinal mucosa must not be associated with irritation in the host, immune activation or with disruption of the natural microbiota. Also, a topical microbicide must be cheap despite having high efficacy. Thus, an urgent need exists to develop efficient systems to allow the delivery of microbicides to mucosal surfaces. Probiotic bacteria have a great potential to serve as a delivery vehicle for microbicide components since they are themselves often able to exclude pathogens in the human host by a number of different activities. Taken together, the use of *L. rhamnosus* GG and GR-1 as delivery vehicles for therapeutic proteins is encouraging, and this work represents an essential first step for the expression of promising anti-HIV lectins.

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Declarations

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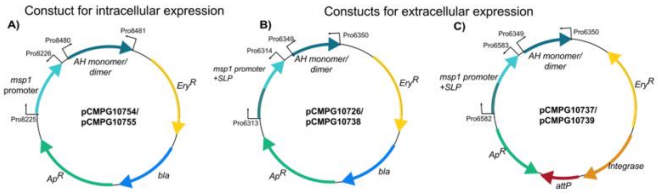

Figure 1. Schematic representation of the plasmids used for intracellular and extracellular expression of the AH monomer and dimer. (A) pCMPG10754/pCMPG10755 were constructed to produce the AH monomer and the AH dimer without the N-terminal His_6-Tag under the control of the *msp1* promoter intracellularly in *L. rhamnosus* GG and GR-1. (B) pCMPG10726/pCMPG10738 were constructed to produce the AH monomer and dimer under the control of the *msp1* promoter extracellularly by *L. rhamnosus* GG and GR-1 by including a secretion signal. (C) pCMPG10737/pCMPG10739 were constructed to achieve a stable extracellular expression of AH monomer and dimer under the control of the *msp1* promoter by *L. rhamnosus*.

Integrase and *attP* genes important for integration of the construct in the host genome; Primers that can be used to check the correct orientation of the *AH* genes are depicted. *ery*^{r}: resistance cassette erythromycin; *bla*: origin of replication; *ap*^{r}: resistance cassette ampicillin

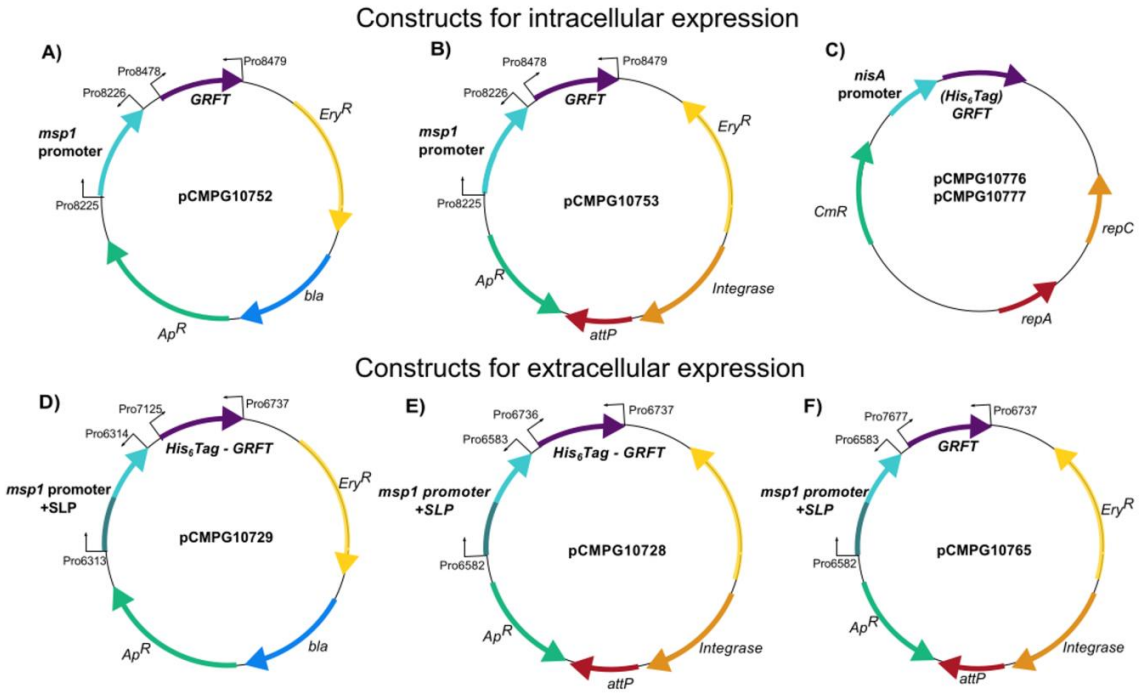

Figure 2 Designed plasmids for expression of recombinant GRFT intra- and extracellularly

(A) Schematic representation of plasmid pCMPG10752 constructed to produce GRFT without N-terminal His_6-Tag intracellularly in *L. rhamnosus* under the control of the *msp1* promoter. (B) Schematic representation of plasmid pCMPG10753 constructed to produce GRFT without N-terminal His_6-Tag intracellularly in *L. rhamnosus* under the control of the *msp1* promoter. (C) Schematic representation of plasmids pCMPG10776 and pCMPG1077 constructed to produce GRFT either with or without N-terminal His_6 tag, respectively, under the control of the *nisA* promoter. (D) Plasmids pCMPG10729 constructed to produce GRFT with a N-terminal His_6 tag (E) Plasmids pCMPG10728 constructed to produce GRFT with an N-terminal His_6 tag. (F) Plasmids pCMPG10765 constructed to produce GRFT without N-terminal His_6-Tag in *L. rhamnosus*.

Primers that can be used to check the correct orientation of the GRFT genes are depicted. *ery*^{r}: resistance cassette erythromycin; *bla*: origin of replication; *ap*^{r}: resistance cassette ampicillin, *cm*^{r} resistance cassette chloramphenicol

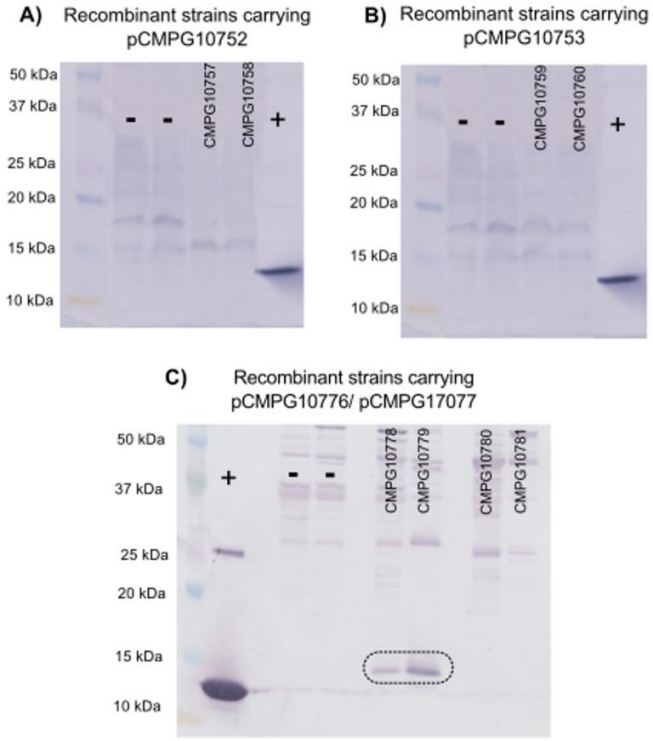

Figure 3. Intracellular expression of GRFT under the control of *msp1* and *nisA* promoters. (A) Cytosolic protein fractions of CMPG10757 (*L. rhamnosus* GG) and CMPG10758 (*L. rhamnosus* GR-1); (B) Cytosolic protein fractions of CMPG10759 (*L. rhamnosus* GG) and CMPG10760 (*L. rhamnosus* GR-1) for detection of recombinant GRFT. (C) Protein fractions of CMPG10778 (*L. rhamnosus* GG), CMPG10779 (*L. rhamnosus* GR-1), CMPG10780 (*L. rhamnosus* GG) and CMPG10781 (*L. rhamnosus* GR-1). For Western blots, strain CMPG10769 (*L. rhamnosus* GG carrying empty pCMPG10724) and CMPG10771 (*L. rhamnosus* GR-1

carrying empty pCMPG10724) were used as negative controls, indicated with the sign minus "–" on the figure. A native GRFT at concentration 5 µg/ml was used as a positive control, indicated with sign plus "+" on the figure. Square boxes represent the recombinant GRFT detected with GRFT specific polyclonal antibodies.

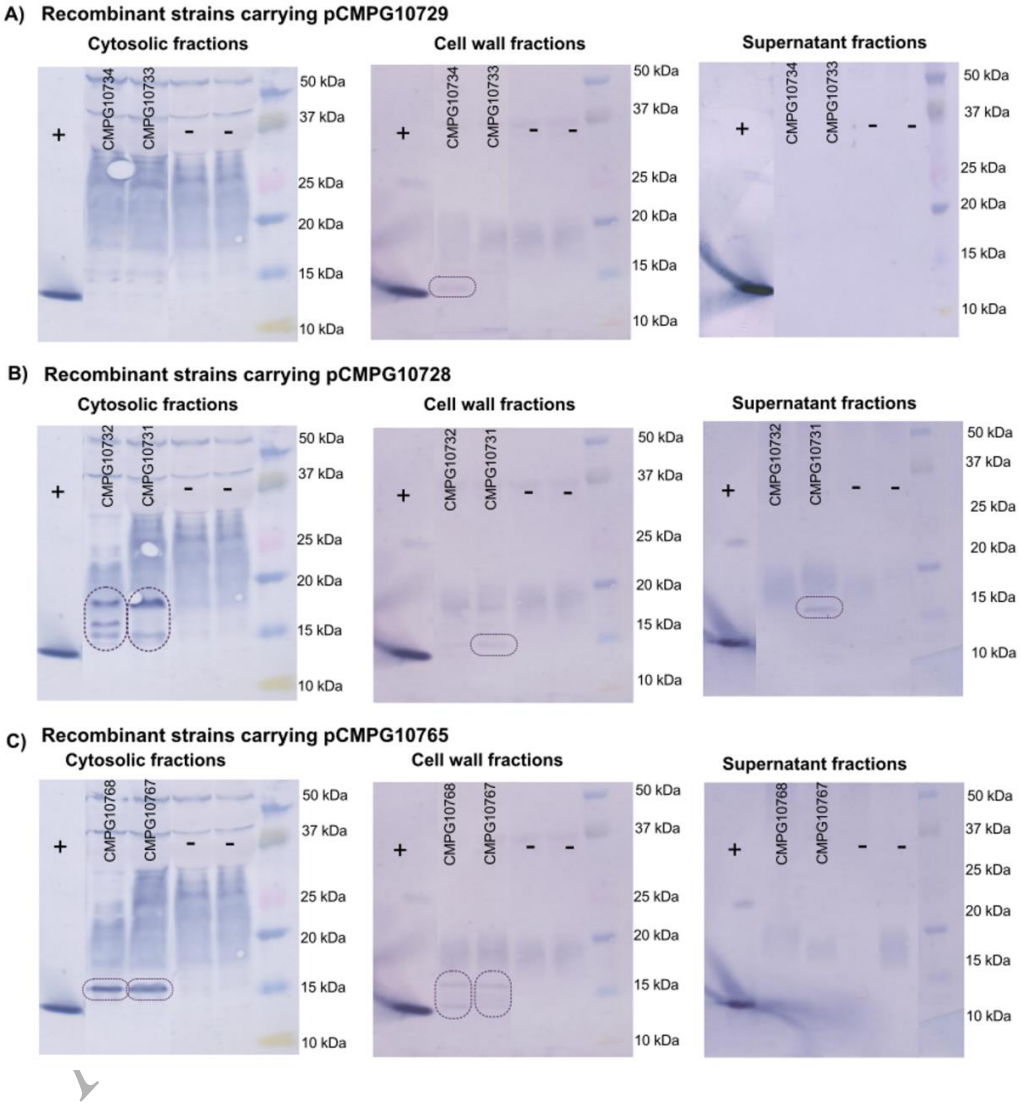

Figure 4. Extracellular expression of GRFT under the control of the *msp1* promoter. (A) Cytosolic, cell wall- and supernatant protein fractions of CMPG10733 (*L. rhamnosus* GG) and CMPG10734 (*L. rhamnosus* GR-1) for detection of recombinant GRFT with a N-terminal His_6

tag. (B). Cytosolic, cell wall and supernatant protein fractions of CMPG10731 (*L. rhamnosus* GG) and CMPG10732 (*L. rhamnosus* GR-1) for detection of recombinant GRFT with an N-terminal His_6-Tag (C). Cytosolic, cell wall and supernatant protein fractions of CMPG10767 (*L. rhamnosus* GG) and CMPG10768 (*L. rhamnosus* GR-1) for detection of recombinant GRFT without an N-terminal His_6-Tag. For Western blots, strain CMPG10769 (*L. rhamnosus* GG carrying empty pCMPG10724) and CMPG10771 (*L. rhamnosus* GR-1 carrying empty pCMPG10724) were used as negative controls, indicated with the sign minus "–"on the figure,and native GRFT (concentration 5 µg/ml) as positive control, indicated with sign plus "+" on the figure. Square boxes represent the detected recombinant GRFT.

Table 1 Detailed description of strains used in this study

Strain name	***Species***	***Vector – self-replicative or integrative***	***Stable or not stable expression***	***Promotor used***	***Recombinant protein***	***With or without N-terminal His_6 Tag***	***Cell localization***
CMPG10731	*L. rhamnosus* GG	pCMPG10728 integrative	Stable	*msp1*	GRFT	With His_6 Tag	Extracellular
CMPG10733	*L. rhamnosus* GG	pCMPG10729 self-replicative	Not stable	*msp1*	GRFT	With His_6 Tag	Extracellular
CMPG10757	*L. rhamnosus* GG	pCMPG10752 self-replicative	Not stable	*msp1*	GRFT	Without His_6 Tag	Intercellular
CMPG10759	*L. rhamnosus* GG	pCMPG10753 integrative	Stable	*msp1*	GRFT	Without His_6 Tag	Intercellular
CMPG10761	*L. rhamnosus* GG	pCMPG10754 self-replicative	Not stable	*msp1*	AH monomer	With His_6 Tag	Intercellular
CMPG10762	*L. rhamnosus* GG	pCMPG10755 self-replicative	Not stable	*msp1*	AH dimer	With His_6 Tag	Intercellular
CMPG10767	*L. rhamnosus* GG	pCMPG10765 integrative	Stable	*msp1*	GRFT	Without His_6 Tag	Extracellular
CMPG10778	*L. rhamnosus* GG	pCMPG10776 self-replicative	Not stable	*nisA*	GRFT	With His_6 Tag	Intercellular
CMPG10780	*L. rhamnosus* GG	pCMPG10777 self-replicative	Not stable	*nisA*	GRFT	Without His_6 Tag	Intercellular
CMPG10732	*L. rhamnosus* GR-1	pCMPG10728 integrative	Stable	*msp1*	GRFT	With His_6 Tag	Extracellular
CMPG10734	*L. rhamnosus* GR-1	pCMPG10729 self-replicative	Not stable	*msp1*	GRFT	With His_6 Tag	Extracellular
CMPG10758	*L. rhamnosus* GR-1	pCMPG10752 self-replicative	Not stable	*msp1*	GRFT	Without His_6 Tag	Intercellular
CMPG10760	*L. rhamnosus* GR-1	pCMPG10753 integrative	Stable	*msp1*	GRFT	Without His_6 Tag	Intercellular
CMPG10763	*L. rhamnosus* GR-1	pCMPG10754 self-replicative	Not stable	*msp1*	AH monomer	With His_6 Tag	Intracellular
CMPG10764	*L. rhamnosus*	pCMPG10755 self-replicative	Not stable	*msp1*	AH dimer	With His_6 Tag	Intracellular

	GR-1						
CMPG10768	*L. rhamnosus* GR-1	pCMPG10765 integrative	Stable	*msp1*	GRFT	Without His_6 Tag	Extracellular
CMPG10779	*L. rhamnosus* GR-1	pCMPG10776 self-replicative	Not stable	*nisA*	GRFT	With His_6 Tag	Intracellular
CMPG10781	*L. rhamnosus* GR-1	pCMPG10777 self-replicative	Not stable	*nisA*	GRFT	Without His_6 Tag	Intracellular

Table 2. Anti-HIV activity of the recombinant GRFT

	HIV-1 NL4.3 (T-tropic virus) EC_{50} [#]	HIV-1 BaL (M-tropic virus) EC_{50} [#]
L. rhamnosus GG WT (negative control)	> 1/25	>1/25
L. rhamnosus GR-1 WT (negative control)	> 1/25	>1/625
L. rhamnosus GG CMPG10778 (GRFT+His_6-Tag)	1/1710* (p =0.02)	>1/605* (p=0.02)
L. rhamnosus GR-1 CMPG10779 (GRFT+His_6-Tag)	1/3021** (p=0.01)	1/1143* (p=0.04)
L. rhamnosus GG CMPG10780 (GRFT-His_6-Tag)	1/56	>1/25
L. rhamnosus GR-1 CMPG10781 (GRFT-His_6-Tag)	1/55	>1/25
GRFT (ng/ml)	< 0.32	0,94

[#]EC50 = effective concentration or concentration required to inhibit HIV-1 replication by 50%. The data represents the average of three independent experiments and reflect the dilution factor of the cytosolic protein extract required to inhibit HIV-1 replication by 50%.

*The dataset comparisons recombinant strains pairwise to wild-type strains (used as negative control) are considered significant ($p \leq 0.05$ indicated with one asterisk in the picture or $p \leq 0.01$ indicated with two asterisks in the figure. The exact p values are also depicted in the table when significance was observed.