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Development of a non-GMO mucosal vaccine against SARS-CoV-2

Steffen Borge
Master of Technology, Chemistry and Biotechnology

Acknowledgments

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Abstract

Lactic acid bacteria (LAB) have been proposed as a good delivery vector of surface-bound antigens, in the development of non-genetically modified organism (non-GMO) mucosal vaccines. LABs are considered good because they have been used in the food industry for centuries and are considered safe for consumption. In addition, LABs can survive, and are found naturally, in the gastrointestinal tract of humans, where they interact with the immune cells in mucosal surfaces. *Lactiplantibacillus plantarum* (previously known as *Lactobacillus plantarum*), have been shown to be one of the most promising LAB candidates.

In this study, two different approaches were attempted to express the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) antigen RBD. 1) The constitutive expression system pOST in *Pichia pastoris*, where the expressed protein contained either a monomer or dimer of RBD. 2) The inducible expression system pSIP in *L. plantarum*. In addition, the antigens were displayed on the surface of *L. plantarum* using the cell wall binding LysM anchor.

The pOST expression system in *P. pastoris* was not able to express the SARS-CoV-2 antigen RBD in this study. Multiple attempts of detection and purification showed no positive results. The pSIP expression system was successful in the expression of the SARS-CoV-2 antigen RBD and displayed the antigens on the surface of *L. plantarum*. Furthermore, binding studies with crude protein extract containing the RBD antigens and *L. plantarum* cells showed positive results in flow cytometry analysis, indicating successful binding of RBD antigens to the bacterial surface.

The current results show a promising starting point towards the development of a non-GMO mucosal vaccine against SARS-CoV-2, using the pSIP expression system in *L. plantarum*.

Sammendrag

Melkesyrebakterier har blitt foreslått som gode kandidater for leveransen av overflate-bundet antigener, i utviklingen av ikke-genetiske modifiserte organisme (non-GMO) slimhinne vaksine. Melkesyrebakterier er ansett som gode kandidater, fordi de har blitt brukt i matindustrien i århundrer og er ansett som trygge å innta. I tillegg kan melkesyrebakterier overleve, og er funnet naturlig, i mage-tarm kanalen hvor de interagerer med immunceller i slimhinnene. *Lactiplantibacillus plantarum* (tidligere kjent som *Lactobacillus plantarum*), har vært vist å være en av de mest lovende kandidatene.

I denne studien ble to framgangsmåter for å uttrykke SARS-CoV-2 antigenet RBD, forsøkt. 1) Det konstitutive uttrykningssystemet pOST i *Pichia pastoris*, hvor det uttrykte proteinet inneholdt enten en monomer eller dimer av RBD. 2) Det induserbare uttrykningssystemet pSIP i *L. plantarum*. I tillegg ble antigenet eksponert på overflaten av *L. plantarum* ved bruk av det cellevegg-bindene LysM ankeret.

I denne studien var ikke uttrykningssystemet pOST i *P. pastoris* istand til å uttrykke SARS-CoV-2 RBD antigenet. Mange forsøk på deteksjon og rensing av antigenet ga ingen positive resultater. pSIP uttrykningssystemet i *L. plantarum* derimot, ble vist til å kunne uttrykke RBD antigenet, i tillegg til antigenet ble eksponert på overflaten av *L. plantarum*. Bindingsstudier av rå protein ekstrakt som inneholdt RBD antigenet og *L. plantarum* celler viste positive resultater etter flow cytometri analyse, noe som indikerer suksessfull binding av RBD antigenet til bakterieoverflaten.

De nåværende resultatene viser en lovende start mot utviklingen av en non-GMO slimhinne vaksine mot SARS-CoV-2, ved bruken av pSIP uttrykningssystemet i *L. plantarum*.

Contents

1 Introduction

Lactic acid bacteria (LAB) are a group of bacteria found in the human microflora and have been regarded as safe for consumption for many years. Studies have proposed the use of LAB as live bacteria vaccine vectors, as some have shown adjuvant properties, as well as their natural ability to survive in the human gastrointestinal tract.

Most pathogens enter the human body through the mucosal pathways. Studies have shown that mucosal vaccines give a more effective immune response in the mucosal layer than traditional injected vaccines.

In this study, the yeast *Pichia pastoris*, and *Lactiplantibacillus plantarum*, is genetically modified to produce severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) antigen, to produce possible non-GMO vaccine candidates using *L. plantarum* as a live delivery vector. The work with the non-GMO vaccine using *P. pastoris* is a part of international project, whereas the work using *L. plantarum* as both a protein expression host and delivery vector, is new.

SARS-CoV-2 is included in this study because of the then ongoing pandemic COVID-19, which has claimed over 6 million lives in three years.

1.1 Lactic acid bacteria

Lactic acid bacteria (LAB) are a group of Gram-positive, non-sporulating bacteria in the shape of rods or cocci (Slover & Danziger, 2008). Bacteria that test positive after the Gram staining procedure generally have the following characteristics: A thick peptidoglycan layer; a cytoplasmatic lipid membrane; as well as having peptidoglycan chains that are cross-linked, creating a rigid cell wall.

LAB is seen as "Generally Recognized as Safe" (GRAS) by the American Food and Drug Administration (FDA), meaning that it is safe for consumption for humans. They play a large role in the production and preservation of food, as the end-product in the metabolic pathway is lactic acid, which leads to acidification and inhibits spoilage in food. LAB is therefore found in milk and dairy products, as well as in other food groups as meats and vegetables. They are also natural inhabitants of the gastrointestinal tract and in mucosal surfaces, and are not usually considered to be pathogenic (Slover & Danziger, 2008). Some LAB have adjuvant properties, meaning they may contribute to an enhanced immune response when used as vectors for vaccine delivery (Wyszynska et al., 2015).

Lactiplantibacillus is a genus of LAB, found in significant amounts in human and animal microbiota (Duar et al., 2017). *Lactiplantibacillus plantarum* (previously named *Lactobacillus plantarum* (Zheng et al., 2020)) is one of the 17 species found in the genus *Lactiplantibacillus. L. plantarum* has GRAS status and is one of the most studied species in regards in DNA- and protein vaccines (Kuczkowska et al., 2019). *L. plantarum* is a good vaccine candidate because of its immunomodulatory effects and adjuvant properties.

A strain of *L. plantarum*, WCFS1, had in 2003 its entire genom sequenced (Kleerebezem et al., 2003). This strain contains many transport and regulatory functions, something that could explain *L. plantarum's* high flexibility and adaptability.

Multiple studies have been done using *L. plantarum* as a vaccine vector and have shown promising results (Kuczkowska et al., 2019; Michon et al., 2016).

1.1 Inducible gene expression system

Inducible gene expression systems allow researchers to control the expression of genes of interest via external stimuli (Sørvig et al., 2003). One of the most used expression systems in LAB is based on the autoregulatory properties of the nisin gene cluster in *Lactococcus lactis*, also called the NICE-system. However, a study done by Sørvig et al. (2003) showed that the two-plasmid NICE-system was not optimal for *L. plantarum*, as the results showed large basal gene expression activity without the induction of nisin.

An inducible expression system has been developed for use in *Lactobacillus sakei* and *L. plantarum*, called the pSIP system (Sorvig et al., 2005; Sørvig et al., 2003). This system is a one-plasmid, inducible expression system based on the promotors and regulatory genes responsible for producing the bacteriocin sakacin A and sakacin P. The pSIP vectors are described as easy-to-use, easy to transform, and should function in any strain of *L. sakei* and *L. plantarum*. The vectors consisting of cassettes with restriction sites and by restriction enzyme digestion and ligation, researchers can therefore easily exchange components in the vectors (Figure 1.1). The pSIP system consists of genes encoding a histidine kinase, a response regulator protein, and the promoter for these genes (Figure 1.1). The gene (*sppIP*) which originally encoded the inducer peptide (SppIP) is deleted from the vector, granting full control over the production of the target protein. When SppIP is added, the histidine kinase protein becomes phosphorylated; the phosphate group is then transferred to the response regulator protein. The phosphorylated response regulator protein then binds to the inducible promotors located up- and downstream of the genes encoding the histidine kinase and response regulator protein, leading to over expression of the target gene. The expression of the target gene leads to more production of histidine kinase- and response regulator proteins, resulting in a positive-feedback-loop and a large amount of the target protein.

Mathiesen et al. (2008) later modified the pSIP-system for the secretion of heterologous proteins in *L. plantarum WCFS1* by using homologous signal peptides. Another modification was made to allow cell surface anchoring of heterologous proteins (Fredriksen et al., 2012; Kuczkowska et al., 2017).

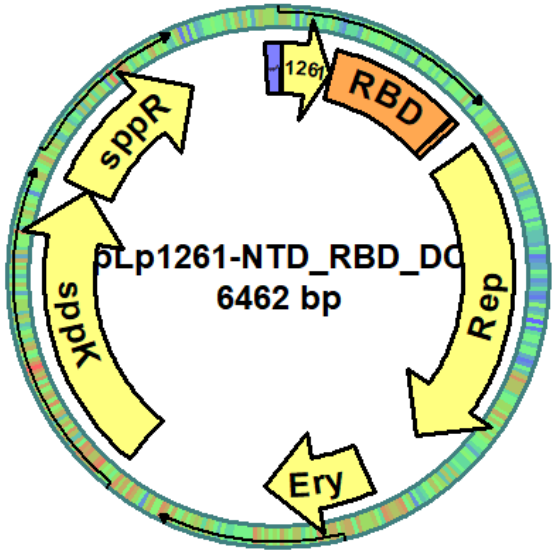


Figure 1.1. A representation of the expression vector of pSIP. sppA (purple block: inducible promotor; Rep: replicon; Ery: Erythromycin resistance marker; sppK; histidine protein kinase; sppR: response regulator.

1.1.1 Pichia pastoris

Yeast cells are widely used in the expression of proteins in pharmaceutical and vaccine production. Compared to bacterial cells, yeast cells have many advantages: Growth speed, posttranslational modification, easy genetic manipulation, and secretory of target protein (Karbalaei et al., 2020). *Pichia pastoris* is a species of methylotrophic yeast, using methanol as its source of carbon and energy, and have become popular stemming from its low cost and efficient expression system. *P. pastoris* can produce high yield of recombinant proteins with similar glycosylation patterns to mammalian cells, as well as appropriate protein folding in the endoplasmic reticulum.

The genetically modified *P. pastoris* strain GS115 is widely used in medical fields and industry, because unlike the wildtype, GS115 uses methanol as its sole source of carbon

and energy (Karbalaei et al., 2020). The most common inducible expression system for *P. pastoris* is using the promotor P_{AOX1} and the α-mating signal of *Saccharomyces cerevisiae*, in the expression of recombinant proteins, using methanol as the inducer (Rieder et al., 2021).

In a study done by Rieder et al. (2021), they devised a way to use *P. pastoris* to produce lytic polysaccharide monooxygenases (LPMOs), enzymes that catalyzes the glycosidic bonds of sugar polymers such as chitin and cellulose. In the production of recombinant LPMOs, the α-mating factor were not suitable, and thus presented a LPMO-tailored constitutive expression system that allowed for an easy and efficient protein expression and a one-step purification, without the use of methanol inducement. An earlier study had identified the promotor P_{GCW14} which could be used for high-level expression of heterologous proteins (Liang et al., 2013). The same promotor was tested in the *P.* pastoris strain pBSYPG11 for LPMOs expression in the study done by Rieder et al. (2021) and showed a significant higher yield than other promotors that were tested.

1.2 Bacteria as delivery vectors

Vaccines are biological products used to induce a non-harmful immune response to give protection against diseases and infections after exposure to certain pathogen (Pollard & Bijker, 2021). The vaccine often contains antigens derived from the pathogen itself or produced synthetically. Vaccines can traditionally be classified as non-live or live, distinguished by if the vaccine contains killed whole organisms, or replicating strains that have their virulence reduced (attenuated). Other classifications of vaccines have later been developed, depending on virulence vectors: nucleic acid-based RNA, DNA, and virus-like particles.

Food-grade bacteria have been proposed as delivery vectors for mucosal vaccine delivery (Pollard & Bijker, 2021; Villena et al., 2021). Using live bacteria have been deemed safer than traditionally live attenuated vaccines, as it removes the possibility for uncontrolled replication in immunocompromised individuals. The microbiota of the intestines has a large role in maintaining the antiviral immunity of mucosal tissues, and its activity. The bacteria *L. plantarum* have been proposed as an ideal vaccine delivery vector, as it has

GRAS status, already been found the human microflora, and have shown adjuvant properties. Multiple studies using *L. plantarum* as a delivery vector have shown very promising results (Kuczkowska et al., 2019; Michon et al., 2016).

Pathogens invade the human body mostly through mucosal surfaces. Vaccines are traditionally injected into muscles; however, studies have shown that delivering vaccines through the mucosal pathways gives better immunological effects in the mucosal layers (Pollard & Bijker, 2021; Villena et al., 2021).

1.2.1 Anchoring of antigen in lactic acid bacteria

For the purpose of developing a non-GMO mucosal vaccine, one strategy utilizes proteins translationally fused with a signal peptide. Signal peptides ensures that the protein is secreted to its designated location and anchored there. Depending on the type of anchor used, the protein is anchored to the cell membrane or cell wall of the bacteria, and thus exposed to surface environment of the cell.

There are four main surface anchors used to anchor heterologous proteins (Figure 1.2). By fusing a N-terminal transmembrane anchor (not illustrated), lipoprotein anchor, LPxTG anchor, or a LysM anchor (Michon et al., 2016; Visweswaran et al., 2014). The lipoprotein and N-terminal transmembrane anchor are anchored to the cell membrane, whereas the LPxTG and LysM anchor are anchored to the peptidoglycan in the cell wall. The LysM anchor is used in this study.

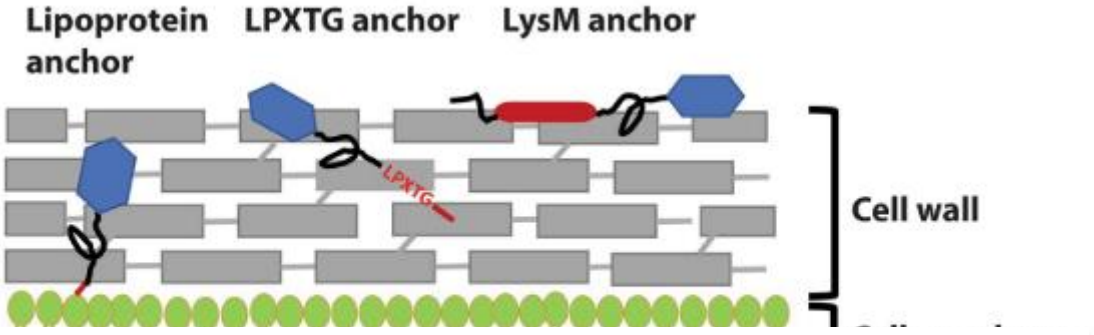


Figure 1.2 An overview of different surface anchors of *L. plantarum.* The blue color are fused antigens, red indicates anchoring domains and motifs, and black shows linker regions. Figure adapted from Mathiesen et al., 2020.

Proteins containing the lysin motif (LysM) are often secreted and non-covalently bound to the peptidoglycan layer of the cell, in both eukaryotes and prokaryotes (Visweswaran et al., 2014). The LysM domain consists of a small repeating LysM sequence, where a single copy of the domain consists of 44 – 65 amino acids and are often found in the N – or C - terminal ends of the protein. It specifically interacts non-covalently to the N-acetylglucosamine monomers in the peptidoglycan layer of the cell, which is also present in chitin. Proteins containing LysM in bacteria are generally bound to newly synthesized peptidoglycan, meaning sites of growth and cell division, which is also closely located to the location where proteins are secreted (Buist et al., 2008).

1.3.2 Non genetically modified vaccines

The use of organisms that have been genetically modified (GMO) in applications that may lead to unsupervised release into the environment is not desirable (Bosma et al., 2006). Heterologous protein display on the surface of bacteria have been suggested in the development of live vaccine delivery systems, often having the expressed protein anchored to the producer cells, thus making the bacteria a GMO. To avoid using GMOs in the final step of the vaccine delivery system, multiple strategies have been developed.

Bosma et al. (2006) describes a novel display system using particles of non-living, non-GMO gram-positive bacteria cells, called gram-positive enhancer matrix (GEM). GEM particles were used as a substrate to bind added proteins via a binding domain derived from *Lactococcus lactis* and tested in nasal vaccine applications. Liu et al. (2017) later tested a GEM based oral vaccine against *Helicobacter pylori,* that they conclude enhanced the efficiency of the vaccine.

The use of the noncovalent binding properties of LysM domains to display proteins on bacterial surfaces have also been suggested as a method to develop non-GMO vaccines (Visweswaran et al., 2014). Multiple studies have successfully exploited this characteristic of LysM domains to create non-GMO vaccine carriers (Moeini et al., 2011; Raha et al., 2005; Xu et al., 2011).

1.3 COVID-19

The COVID-19 pandemic refers to an outbreak the seventh human coronavirus, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), first identified in Wuhan, China in 2019. It has of this moment resulted in 6,2 million deaths worldwide according to WHO, and its regular symptoms are: fever, dry couch, muscle pain, fatigue, and shortness of breath (Yang et al., 2020).

Coronaviruses are positive-sense single-stranded RNA viruses of the genus *Betacoronavirus* (Villena et al., 2021). The SARS-CoV-2 viral genome of 29,9 Kb consists of four structual proteins: spike, envelope, membrane and nucleocapsid proteins, as well as other non-structural proteins (Figure 1.3). The receptor-binding domain (RBD) is located between the two spike sub-domains (S1 & S2) of the spike domain (Villena et al., 2021). The spike protein is responsible for the entry into host cells to sustain person-to-person transmission, where the RBD interacts with the angiotensin converting enzyme 2 (ACE2), which is expressed and found on the surface a wide range of cells. Thus, cells with high expression of ACE2, such as in the nasal and bronchial epithelial, small intestine, liver, and cardiovascular tissue, are more vulnerable for infection. The spike protein is also responsible for the zoonosis ability of the virus, meaning transmission between animals and humans, which is believed to be the source of origin for the SARS-CoV-2 virus (Arashkia et al., 2021). Phylogenetic analysis of the full-length genome indicates that SARS-CoV-2 is related to two types of bat coronaviruses (SL-CoV ZC45 and SL-CoV ZXC21), but more distantly related to SARS-CoV. Another phylogenetic study showed that the RBD sequence of SARS-CoV-2 was more closely related to SARS-CoV, indicating high similarity of the spike gene between the two viruses (Lu et al., 2020).

The currently developing vaccines for SARS-CoV-2 mainly focus on the spike protein, and the RBD within it (Arashkia et al., 2021). There are three vaccines accepted for use in Norway as of May, 2022: Comirnaty, produced by BioNTech/Pfizer, Spikevax by Moderna, and Nuvaxovid by Novavax (Legemiddelverket, 2022). Comirnaty and Spikevax are both mRNA vaccines, where as Nuvavax is a protein subunit vaccine. The mRNA vaccines contains genetically modified mRNA that instructs the cells themselves

to create pieces of the spike protein or the RBD, causing the body to create antibodies. Protein subunit vaccines contains parts of the spike protein, which in turn induces creation of antibodies. Two vaccines using a viral vector (Vaxzevria by AstraZeneca and COVID-19 Vaccine Janssen by Janssen) were previously also accepted for use in the general populace, but were removed as they both shared the same type of negative side-effects (Franchini et al., 2021; Legemiddelverket, 2022).

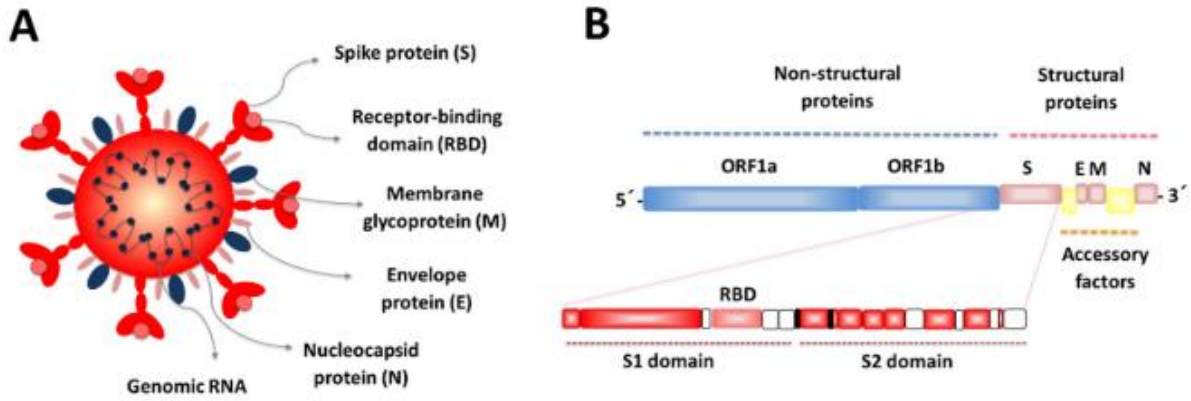


Figure 1.3. Schematic of the SARS-CoV-2 virion (A) and genomic structures (B). (A) The SARS-CoV-2 virion consists of a nucleocapsid of genomic RNA and nucleocapsid protein, which is enclosed in the envelope by the spike – and membrane glycoprotein. (B) Shows the genomic structures, where two-thirds of the genome encodes for non-structural proteins, and the remaining one-third encoding for four different structures: spike (S), envelope (E), membrane glycoprotein (M) and nucleocapsid (N). The spike domain is highlighted, showing the two different spike domains (S1 & S2), with the receptor-binding domain (RBD) in-between. Figure is adapted from (Villena et al., 2021).

1.4 Aim of Study

This study was part of a larger project, with the long-term goal to create a non-GMO mucosal vaccine against SARS-CoV-2, using *L. plantarum* as a delivery vector of antigens. The CoV-2 spike RBD is an attractive target for a vaccine, therefore antigens of RBD was used in this study. Two plasmid constructs were created for protein expression in *P. pastoris*, one containing a monomer of RBD, another an RBD-dimer, as a study showed that a vaccine with RBD-dimer provided a higher protection from infection in mice (Dai et al., 2020). Another approach that was explored used *L. plantarum* as a protein expression host, with the same end-goal of also using *L. plantarum* as a delivery vector.

The experimental approach in this study had the following steps:

- Construction of vectors for production of SARS-CoV-2-RBD, using the LysM anchor for surface display.
- Transforming the constructed vectors into *P. pastoris* and *L. plantarum*
- Disruption of cells, following purification, of the produced proteins.
- Analysis of the surface display of the antigen in *L. plantarum*
- Binding studies of the antigen on the surface of *L. plantarum* and other species.

2 Methods

2.1 Cultivation of yeast

Yeast was cultivated either in solid agar or in liquid medium, with antibiotics added if necessary. *Pichia pastoris* was cultivated in YPD (Sigma-Aldrich) medium with Zeocin (Invitrogen) added. In liquid medium, *P. pastoris* was incubated between 1-4 days in a shaking incubator at 30 ℃, and solid agar medium was incubated at 30 ℃ for 1-4 days without shaking. Zeocin was added at a concentration at 100 µg/mL from frozen stock.

2.1.1 Cultivation of bacteria

LB medium recipe for 1 L:

- Tryptone 10 g
- NaCl 10 g
- Yeast extract 5 g
- dH_2O Up to 1L

Media:

1. Regents were mixed and stirred with a magnet stirrer in a 1 L Duran Bottle
2. Solution was autoclaved at 121 ℃ for 20 minutes.

Agar:

1. Reagents were mixed and dissolved in 1 L dH_2O
2. 1.5 % (w/v) agar was added and dissolved to the mixture.
3. Solution was then autoclaved at 121 ℃ for 20 minutes.
4. The media was cooled to 60 ℃ before potential antibiotics were added and distributed to petri dishes. After the media solidified, the petri dishes were stored at 4 ℃.

Brain-Heart-Infusion (BHI)

Media:

1. 37 g BHI (Oxoid) was dissolved in 1 L dH_2O and autoclaved at 121 ℃ for 20 minutes.

Agar:

1. 37 g BHI was dissolved in 1 L dH_2O. 1.5 % (w/v) agar was added to the solution and dissolved.
2. Solution was then autoclaved at 121 ℃ for 20 minutes.
3. The media was cooled to 60 ℃ before potential antibiotics were added and distributed to petri dishes. After the media solidified, the petri dishes were stored at 4 ℃.

Bacteria was cultivated either in solid agar or in liquid medium, with antibiotics if necessary.

Escherichia coli was grown in LB and BHI medium with either zeocin or erythromycin (Merck) added, from stock. The bacteria in liquid media were incubated in a shaking incubator at 37 ℃, and in solid agar at 37 ℃ without shaking. *Lactiplantibacillus plantarum* was grown in MRS, with erythromycin added if necessary. *L. plantarum* was grown in either liquid media or solid agar, at 30 ℃ without shaking, with antibiotics if necessary (Table 2.1)

Table 2.1. Antibiotic concentration used for *E. coli* and *L. plantarum*.

Antibiotics	**Liquid medium – *E. coli* (µg/mL)**	**Solid medium – *E. coli* (µg/mL)**	**Liquid medium – *L. plantarum* (µg/mL)**	**Solid medium – *L. plantarum* (µg/mL)**
Erythromycin	200	200	10	10

2.2 Storage of yeast and bacteria

When yeast and bacteria was wanted for storage over a long time, 1000 µL of overnight liquid culture was mixed in a cryovial with 300 µL sterile 87% glycerol (Merck), inverted a couple of times for a homogeneous solution and stored at -80 ℃. Glycerol was added to the liquid culture to provide protection for the bacteria cells from damage from low temperatures.

To cultivate a yeast or bacteria from long-time storage, one would under sterile conditions use a toothpick to pick up a small amount of frozen culture, and drop the toothpick in the appropriate growth medium, with antibiotics if necessary, and handled as stated in 2.1.1.

2.3 Isolation of plasmids

The Nucleospin® Plasmid Kit (MACHEREY-NAGEL) was used to isolate plasmids from bacteria and yeast. From the protocols provided with the kit, Protocol 5.1 and 5.2 was used, dependent on if the plasmid was a low-copy or high-copy.

2.3.1 Plasmids

Table 2.2. Plasmids used in this study.

Plasmid	**Description**	Source
pEV	pSIP401 derivative without any target genes. Empty vector.	(Fredriksen et al., 2012)
pBSYGCW$_{14}$Z	Constructed plasmid utilizing the pOST expression system.	(Rieder et al., 2021)
pBSYGCW$_{14}$Z -Ost-1_LysM_RBD_DC (LysM_RBD)	Derivative of pBSYGCW$_{14}$Z where the stuffer fragment was replaced with SARS-	This work

	CoV-2-RBD monomer and LysM.	
pBSYGCW$_{14}$Z -Ost-1_LysM_scRBD_DC (LysM_scRBD)	Derivative of pBSYGCW$_{14}$Z where the stuffer fragment was replaced with SARS-CoV-2-RBD dimer and LysM.	This work
pLp_1261_RBD_DC (pLp1261_RBD)	pSIP401 derivative to produce RBD with the Lp_1261 lipoprotein anchor sequence.	(Trondsen, 2021)
pLp_3050_DC_RBD_cwa3001 (pLp3001_RBD)	pSIP401 derivative to produce RBD.	(Trondsen, 2021)
pLp_3014_Ag85B_ESAT6_DC (pLp_3014_Ag85)	pSIP401 derivative for production of Ag85B-ESAT6 with Lp_3014 LysM-anchor.	(Målbakken, 2014)
pLp_3014_LysM_RBD_DC (pLp3014_LysM_RBD)	Derivate of pLp_3014_Ag85 with Ag85B_ESAT6 replaced with RBD.	This work
pSIP3014_LysM_RBD_DC_His (pSIP3014_LysM_RBD)	Derivate of pLp_3014_Ag85 with Ag85B_ESAT6 replaced with RBD and a His-tag.	This work

2.4 DNA and protein concentration

2.4.1 DNA concentration

Materials:

Qubit® dsDNA BR Assay Kit (Invitrogen)

Qubit® Fluorometer (Invitrogen)

DNA

Procedure

1. Qubit Reagent was diluted 1:200 with Qubit BR Buffer in room temperature and mixed for a homogenous solution.
2. 2 µL of DNA sample was added to 198 µL solution from (1), vortexed and quickly spun in a bench centrifuge.
3. The Qubit® Fluorometer was calibrated using Standard 1 and Standard 2 (values stored on the fluorometer) before DNA concentration was determined.

3.2 If the measured DNA value went over the values of Standard 1 and Standard 2, the added DNA sample was lowered, i.e., 1 µL to 199 µL solution from (1).

2.4.2 Bradford protein assay

To measure the amount of protein in a sample, a Bradford protein assay was performed.

Materials

BSA Standard (Sigma)

1x Protein Assay Dye Reagent (BioRad)

PBS

1 mL cuvette

Procedure

1. 1-20 µL of sample and 780 – 799 µL PBS was mixed in an Eppendorf tube. Total volume of sample and PBS was always 800 µL.

2. At least one parallel of each sample was made.
3. 200 µL of Dye Reagent was added to the sample and vortexed well, before incubated at room temperature for at least 5 minutes, but no longer than 1 hour.
4. OD_{595} was then measured using PBS as blank first, before the sample and its parallel.
5. From the measured value, the protein concentration in the sample was calculated.

2.5 Restriction enzyme digestion of DNA

When adding or removing DNA fragments from plasmids, restriction enzymes were used to cut open the plasmid. Different restriction enzymes cut double stranded DNA at certain places, where the restriction enzyme finds its recognition sequences, leaving a clean break, or sticky-end/overhang. Depending on the goal of the digestion, one or two restriction enzymes were added to the DNA sample. If two restriction enzymes were used at the same time, compatibility between their temperature of digestion and buffer were necessary.

Materials

DNA

Restriction enzyme

Compatible buffer

dH_2O

Procedure

1. The different components were mixed at room temperature in an Eppendorf tube (Table 2.3).
2. The Eppendorf tube was incubated for 1-3 hours in a water bath, holding the appropriate temperature for the restriction enzymes.
3. After digestion, the sample was loaded on an agarose gel.

Table 2.3. The required components for restriction enzyme digestion of DNA.

Component	**Volume (µL)**
DNA	X
Restriction enzyme	5
Buffer	5
dH_2O	Up to 50

2.6 Polymerase chain reaction

Polymerase chain reaction (PCR) is a technique used to amplify specific segments of DNA. During the procedure, the DNA sample is exposed to different temperatures, causing the DNA to split into single strands (denaturation), primers to bind to a specific DNA segment (annealing), and the complementary DNA strand to be synthesized by DNA polymerase (elongation). This process is then repeated multiple times, leading to many copies of the specific DNA segment.

In the denaturation stage, the temperature rises to 98 ℃, causing the hydrogen bonds between the nucleotides between the DNA to break, forming single-stranded DNAs. In the annealing stage, the temperature is reduced to 50-72 ℃, depending on the primers used in PCR. This enables the primers to bind to the single-stranded DNA segments. During the elongating stage, the temperature is kept at 72 ℃, so that the added DNA polymerase can synthesize the complementary DNA strand of the single-stranded, primer-bound DNA segment, using deoxynucleotides added with the DNA polymerase. This cycle of temperature changes is repeated 25-35 times.

The primers used in this study are shown in Table 2.4. The primers were used in PCR reactions in 2.6.1 and 2.6.2, In-fusion cloning 2.9.3 and sequencing.

Table 2.4. Primers used in this study.

Name	Sequence	Description
SekF	GGCTTTTATAATATGAGATAATGCC	Forward primer for all sequencing of pSIP derivatives
SekR	CCTTATGGGATTTATCTTCCTTATTCTC	Revers primer for all sequencing of pSIP derivates
LysM_RBD_His_F	TATGATTCACATATGACTTACACCGTTAAGAGCGGT	In-fusion forward primer for insertion of LysM and RBD antigens into pSIP3014_LysM_RBD
LysM_RBD_His_R	ATTTGAAGCTAAGCTTTCAATGATGATGATGATGATGTGAACCTGGACGCTGTGGGGTTGAA	In-fusion reverse primer for insertion of LysM and RBD antigen into

		pSIP3014_LysM_RBD

2.6.1 Q5® High-Fidelity DNA polymerase

Materials

Q5® Hot Start High-Fidelity 2X Master Mix

Template DNA

Primers

dH_2O

0,2 mL PCR tubes

PCR machine

Procedure

1. Components (Table 2.5) were mixed in PCR tubes, all kept on ice, and if necessary, the PCR tubes would be quickly spun in a bench centrifuge to collect all liquid.
2. The PCR tubes were placed in the PCR machine and followed the program from table 2.6.

Table 2.5. PCR components using Q5® High-Fidelity DNA polymerase

Component	**Volume (µL)**

Q5® Hot Start High-Fidelity 2X Master Mix	25
10 µM Forward primer	2,5
10 µM Reverse primer	2,5
Template DNA	1
dH_2O	Up to 50

Table 2.6. Program of thermocycling used for Q5® High-Fidelity DNA polymerase.

Step	**Temperature (°C)**	**Time (s)**	**Cycles**
Initial denaturization	98	30	1
Denaturization	98	10	25-35
Annealing	50-72*	30	
Elongation	72	20-30/Kb**	
Final elongation	72	120	1
Hold	4	∞	

* Varies depending one the primers used in the PCR.

** Varies depending on the length of the DNA segment that amplified. Per 1000 base pairs of the DNA segment, 20-30 seconds is the recommended time.

2.6.2 Taq DNA Polymerase

To check for a successful transformation of plasmid in bacteria and yeast cells, grown on solid agar, Red Taq PCR was used. A small piece of a colony was transferred to an empty PCR tube via a toothpick, gently scraped against the insides of the tube for the release of the sample and acting as the template DNA in a usual PCR reaction. This was done before adding the other components (Table 2.7).

Materials

RED Taq DNA Polymerase Master Mix

Template DNA

Primers

dH_2O

0,2 mL PCR tubes

PCR machine

Procedure

1. Components (Table 2.7) were mixed in PCR tubes, all kept on ice, and if necessary, the PCR tubes would be quickly spun in a bench centrifuge to collect all liquid.
2. The PCR tubes were placed in the PCR machine and followed the program from table 2.8.

Table 2.7. Components of Red Taq DNA polymerase.

Component	**Volume (µL)**
RED Taq DNA Polymerase Master Mix	25
10 µM Forward primer	2,5
10 µM Reverse primer	2,5
Template DNA	*
dH_2O	Up to 50

* Since the amount of sample picked via a toothpick is almost negligent, it was counted as zero volume added to the reaction.

Table 2.8. Program for thermocycling conditions for Taq DNA polymerase.

Step	**Temperature (°C)**	**Time**	**Cycles**
Initial denaturization	95	2 minutes	1
Denaturization	95	30 seconds	25-35
Annealing	50-65*	30 seconds	
Elongation	72	1 minute/Kb**	
Final elongation	72	5 minutes	1
Hold	4	∞	

* Varies depending on the primers used in the PCR.

** Varies depending on the length of the DNA segment that amplified. It is recommended that for each 1000 base pair DNA, 1 minute is needed.

2.7 Agarose gel electrophoresis

Agarose gel electrophoresis is a method to separate macromolecules such as DNA or proteins in a sample, in a matrix of agarose. The macromolecules are separated by adding a voltage through the gel, leading them towards the positive charge, since DNA has a negative charge. Depending on the size of the macromolecules, i.e., the size of a DNA fragment, they will separate and travel faster or slower through the agarose matrix, since the negative charge of the DNA is the same regardless mass. Loading dye and peqGREEN are added to the sample to make the sample visible on the gel, and a standard ladder with known sizes are added to a well such that the sizes of our samples can be determined.

Materials

SeaKem® LE Agarose

PeqGREEN

Loading dye

DNA ladder

1x TAE Buffer (BioRad)

GelDoc EZ Imager

Procedure

1. To make a stock solution readily available for agarose gel electrophoresis:
    a. 6 g SeaKem® LE Agarose was dissolved in 0,5 L 1x TAE Buffer in a Duran Bottle and mixed with a magnet stirrer.
    b. The solution was autoclaved at 115 ℃ at 20 minutes and stored at 60 ℃.

2. 60 mL of warm stock solution was mixed with 2,5 µL peqGREEN and poured onto an empty molding tray with a comb and fitted onto a tray to prevent leakage of the solution.
3. The gel would take 15-20 minutes to turn solid, and the comb would be removed.
4. The solid gel was transferred to a electrophoresis chamber, and 1x TAE Buffer was added to the chamber such as it covered the gel and empty wells.
5. Samples with added 5 µL Loading dye was loaded into the empty wells, as well as a ladder.
6. Gel runtime used differed depending on the strength of the voltage and length of DNA fragments loaded onto the gel. Usually, 90 V for 45 minutes was used.
7. After ended runtime, the gel was removed from the electrophoresis chamber, and placed on an appropriate tray complementary to the type of gel electrophoresis used, in the GelDoc EZ Imager machine.
    a. If the separated DNA fragments were to be used later, the bonds were exercised from the gel with a scalpel and stored in -20 ℃ or used directly afterwards.

2.7.1 Gel electrophoresis of proteins

When separating proteins based on their molecular weight using gel electrophoresis, Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) is often used. The protein samples are denatured by the addition of lithium dodecyl sulphate (LDS), dithiothreitol (DTT) and incubated at approximately 100 ℃. The LDS breaks non-covalent bindings in the proteins, unfolding them, and binds to them to prevent refolding, as well as lowers the proteins intrinsic charge. DTT is added to break disulfide bridges in the protein. With LDS bound to them, proteins will have a negative charge that will lead the proteins through the gel towards the positive charged anode when voltage is added to the system. Depending on the size of the proteins, the lengths they will travel in the gel differs. Lower sized proteins will travel further in the gel than larger ones, since the polyacrylamide matrix becomes tighter the further the proteins travel.

Materials

Novex® NuPAGE® SDS-PAGE Gel System

Appropriate ladder

1x TGS-Buffer

Protein sample

LDS Sample Buffer (4x)

Reducing Agent (10x)

NuPAGE® Novex Bis-Tris gel

Procedure

1. A working solution was made with 7,5 µL LDS Sample Buffer and 3 µ L Reducing Agent.
2. 10 µL of working solution was added to 20 µL protein sample and incubated at 100 ℃ in a water bath or heating block, for 10 minutes.
3. NuPAGE® Novex Bis-Tris gel was fitted in the electrophoresis chamber, before TGS-Buffer was added in both the chamber and placeholder for the gel.
4. Samples was then loaded into the wells of the gel with a ladder.
5. The length of time and voltage used differed, but usually 30 minutes at 200 V was used.

2.7.2 Methanol/ Chloroform protein precipitation

Materials

Methanol

Chloroform

dH_2O

Procedure

1. 400 µL methanol was added to 100 µL sample in a Eppendorf tube and vortexed well.
2. 100 µL chloroform was added to the sample and vortexed well.
3. 300 µL dH_2O was added to the sample and vortexed well.
4. The sample was centrifuged by 14.000 x g for 2 minutes.
5. The top aqueous layer in the tube was pipetted off. There should a thin layer separating the top and bottom of liquid, where the protein lies.
6. 400 µL of methanol was added to the sample and vortexed well.
7. The sample was centrifuged by 14.000 x g for 3 minutes.
8. The supernatant was pipetted off as much as possible without disturbing the pellet.
9. The sample was placed in a Speed-Vac to dry the sample as much as possible without drying out the pellet too much.
10. Sample was then stored at -20 ℃.

2.8 DNA extraction and purification from agarose gels

To extract and purify DNA and PCR amplified DNA from agarose gels, the NucleoSpin® Gel and PCR Clean-Up Kit was used. Depending on whether the DNA was extracted or the PCR amplified DNA was to be purified, the protocols provided by the manufacturer was Protocol 5.2 or 5.1.

2.9 Ligation

Ligation is used during the process of cloning recombinant DNA into vectors. An added ligase enzyme is used to catalyze covalent binding of two complementary DNA segments, forming phosphodiester bonds between the 5'-phosphate and 3'-hydroxyl ends.

2.9.1 Gibson Assembly

The Gibbson Assembly method to assemble DNA fragments and is carried out in three enzymatic reactions. A 5' exonuclease generates long overhangs of 15-20 bp, a DNA polymerase then fills the gaps of the overhangs, and a DNA ligase seals the gaps of the annealed DNA fragments. To calculate the amount of DNA fragments to add to the mix, NEBioCalculator (https://nebiocalculator.neb.com/#!/ligation) was used.

Materials

Gibson Assembly Master Mix (2x)

DNA fragments

dH_2O

Procedure

1. The different components were gently mixed on ice in an Eppendorf tube (Table 2.9).
2. Sample was incubated in a water bath at 50 ℃ for 1 hour and stored at -20 ℃ or kept on ice for further use in transformation.

Table 2.9. Components of a Gibbson Assembly with 2-3 fragments.

Component	**2-3 fragment assembly**
Total amount of DNA fragments	0,02-0,5 pmols*
Gibson Assembly Master Mix (2x)	10 µL
dH_2O	Up to 20 µL

*Optimized cloning efficiency is 50-100 ng of vector with an excess of 2-3 times insert added.

2.9.2 Quick Ligation

Materials

Quick Ligase

Quick Ligase Reaction Buffer (2x)

Vector DNA

Insert DNA

dH_2O

Procedure

1. The molar ratio of vector to insert to be used was calculated using the NEBioCalculator (https://nebiocalculator.neb.com/#!/ligation). Molar ratios of 1:3 – 1:7 was used, with the added vector varied from 50 g to 100 g.
2. The different components were gently mixed in an Eppendorf tube on ice, with Quick Ligase added last (Table 2.10).
3. Incubated for 5 minutes at 25 ℃ in a water bath
4. Stored at -20 ℃ or kept on ice for further use.

Table 2.10. Quick Ligase components representation using a molar ratio of 1:3 of vector to insert DNA.

Components	**20 µL reaction**
Quick Ligase Reaction Buffer (2x)	10 µL
Vector DNA	50 ng *
Insert DNA	37,5 ng *
Quick Ligase	1 µL
dH_2O	Up to 20 µL

*Could change depending on the chosen molar ratio of vector to insert DNA.

2.9.3 In-Fusion Cloning

The In-Fusion cloning method uses the in-fusion enzyme, which fuses a linearized vector to the insert DNA via a 15 base pair overhang on each end of the insert DNA. The DNA fragment is then amplified via PCR with primers designed with the 15 base pair overhang in-mind, with binds to the insert DNA, where the overhang is complementary to the ends of the linearized vector.

Materials

Linearized vector

5x In-Fusion® HD Enzyme Premix

Insert DNA (purified PCR fragment)

dH_2O

Procedure

1. The molar ratios of the vector and insert was calculated using the In-Fusion molar ratio calculator (https://www.takarabio.com/learning-centers/cloning/primer-design-and-other-tools/in-fusion-molar-ratio-calculator), where a 2:1 ratio was used.
2. The components were gently mixed in an Eppendorf tube on ice (Table 2.11).
3. Incubated for 15 minutes at 50 ℃, and then stored at -20 ℃ or kept on ice for further use in transformation.

Table 2.11. Components of the In-Fusion cloning procedure.

Components	**Volume (µL)**
5x In-Fusion® HD Enzyme Premix	2
Vector	50 – 100 ng
Insert	50 – 100 ng

dH_2O	Up to 10

2.10 Electrocompetent *Pichia pastoris*

Cells that can take up free extracellular DNA are called competent cells, and when they do, they can be called transformed. When a cell is called electrocompetent, it means that it takes up the DNA through electroporation, a process where the cell is exposed for an electrical pulse that makes the cell wall permeable.

The method for electrocompetent *P. pastoris* cells are from the protocol described by (Lin-Cereghino et al., 2005).

Materials

YPD

YPD/0,02 M HEPES (Sigma-Aldrich)

1,0 M dithiothreitol (DTT)

dH_2O

1,0 M Sorbitol

Procedure

1. *P. pastoris* from long-time storage was cultured overnight in 50 mL YPD at 30 ℃ with shaking at 225 rpm.
2. The culture was then diluted late the next day, to a OD_{600} of 0,1 in 500 mL YPD, and incubated overnight at 30 ℃ with shaking to an OD_{600} of 1,3-1,5.
3. The culture was centrifuged for 10 minutes at 4000 x g at 4 ℃. All following centrifuge steps held the same speed and temperature.
4. 100 mL of YPD/0,02 M HEPES was added.
5. 2,5 mL 1,0 M DTT was added dropwise.
6. Incubated for 15 minutes at 30 ℃ with shaking.

7. dH_2O was added up to 500 mL.
8. Centrifuged for 10 minutes.
9. Resuspend pellet in 500 mL dH_2O.
10. Centrifuge for 10 minutes.
11. Resuspend pellet in 250 mL dH_2O.
12. Centrifuge for 10 minutes.
13. Resuspend pellet in 20 mL 1,0 M sorbitol.
14. Centrifuge for 10 minutes.
15. Resuspend pellet in 1 mL sorbitol.
16. 40 µL of the resuspended pellet was aliquoted in 1,5 mL Eppendorf tubes kept on ice and stored at -80 ℃.

2.10.1 Electrocompetent Lactiplantibacillus plantarum

L. plantarum was grown in medium containing glycine during the making of electrocompetent cells. Glycine replaces L-alanine in the cell wall during growth, which makes the cell wall more permeable for extracellular DNA uptake.

Materials

MRS

MRS + 1 % glycine

20% glycine

30 % PEG_{1450}

MRSSM

Solutions

30 % PEG_{1450}

- Components:
    - 30 g PEG_{1450} dissolved in 70 mL dH_2O

MRSSM

- Components:
    - MRS
    - 0,5 M sucrose
    - 0,1 M $MgCl_2$
- Mixed and autoclaved at 115 ℃ for 20 minutes and stored at room temperature.

Procedure

1. *L. plantarum* was cultured from long-time storage overnight in 10 mL MRS at 37 ℃ without shaking.
2. 1 mL of the overnight culture was used in a serial dilution of 10^{-1} – 10^{-7} in MRS + 1 % glycine. Incubated overnight at 37 ℃.
3. 1 mL of a culture having an OD_{600} of 2,5 ± 0,5 was diluted in 20 mL MRS + 1 % glycine. This culture was cultured at 37 ℃ until it had an OD_{600} of 0,7 ± 0,7 and placed on ice.
4. The culture was centrifuged at 4250 x g for 10 minutes at 4 ℃.
5. Pellet was resuspended in 25 mL cold, new 30 % PEG_{1450}. The tube was gently inverted and placed on ice for 10 minutes.
6. The culture was centrifuged at 4250 x g for 10 minutes at 4 ℃.
7. Pellet was resuspended in 400 µL 30 % PEG_{1450} and 40 µL was aliquoted in pre-frozen Eppendorf tubes and stored at -80 ℃.

2.11 Transformation

2.11.1 Transformation of chemically Competent *E. coli*

Materials

LB and BHI agar plates with antibiotics

Ligation mix

One Shot ™ TOP10 chemically competent *E. coli* (Invitrogen)

Falcon 2059 Polypropylene Round Bottom tube 14 mL

Procedure

1. A tube of One Shot ™ TOP10 chemically competent *E. coli* was thawed on ice from long-time storage.
2. In a cold Falcon tube on ice, 50 µL of *E. coli* and 1-5 µL of DNA was gently mixed and incubated on ice for 30 minutes.
    a. When the ligation mix from Gibbson Assembly (Methods 2.9.1) was used, the mix was diluted 1:5 with dH_2O before being added to the competent *E. coli*.
3. The bacteria cells were heat-shocked at 42 ℃ for 30 seconds, before being placed on ice again for 2 minutes.
4. 250 µL LB medium was added to the transformation mix and incubated at 37 ℃ for 1-3 hours with shaking at 225 RPM.
5. 100-200 µL of the transformation mix was then spread on LB or BHI plates with appropriate antibiotics added to them and incubated overnight at 37 ℃.

2.11.2 Electrotransformation of *P. pastoris* cells

Materials

YPD agar plates with zeocin

Bio-Rad GenePulser ® II

Bio-Rad Pulse controller plus

Electrocompetent *P. pichia*

Electroporation cuvette, 0,2 cm, Gene Pulser®

DNA

1,0 M sorbitol

Procedure

1. 0,1 µg to a total of 5,0 µL DNA and 40 µL thawed electrocompetent *P. pastoris* cells were mixed and transferred to a cold electroporation cuvette.
2. The electroporator used the following parameters:

   Resistance: 200 Ω

   Capacitance: 25 µF

   Voltage: 1500 V

3. The electroporation cuvette was placed in the electroporator and given an electrical pulse.
4. 1 mL of cold 1,0 M sorbitol was added to the cuvette immediately afterwards, transferred to an Eppendorf tube and incubated for 1-3 hours at 30 ℃.
5. After incubation, 100-200 µL of the transformation mix was spread on YPD agar plates containing 100 µg/ml zeocin and incubated 2-4 days at 30 ℃.

2.11.3 Electrotransformation of *L. plantarum* cells

Materials

Bio-Rad GenePulser ® II

Bio-Rad Pulse controller plus

Electrocompetent *L. plantarum*

Electroporation cuvette, 0,2 cm, Gene Pulser®

DNA

MRSSM

MRS agar plates with antibiotics

Procedure

1. 5 µL DNA was added to 44 µL thawed electrocompetent *L. plantarum* and gently mixed on ice, before transferred to a cold electroporation cuvette.
2. The electroporator used the parameters:

   Resistance: 400 Ω

   Capacitance: 25 µF

   Voltage: 1500 V

3. The electroporation cuvette was placed in the electroporator and given an electrical pulse.
4. 450 µL MRSSM was immediately added to the cuvette and transferred to an Eppendorf tube.
5. The transformation mix was incubated at 37 ℃ for 2-4 hours without shaking.
6. 100-200 µL of the transformation mix was spread on MRS agar plates containing 10 µg/ml erythromycin and incubated over night at 37 ℃.

2.12 Sequencing of isolated plasmid

Isolated plasmids wanted for sequencing was sent to a separate laboratory to undergo Sanger sequencing. 400-500 ng of purified plasmid was added to an Eppendorf tube with 2,5 µL of primer (10 µM), either forward or reverse, and dH_2O up to 11 µL of total volume. The results were analyzed with the software CLC DNA Main Workbench 7.

2.13 Preparation of gene product analyses

2.13.1 *P. pastoris* cultivation and harvesting

Materials

YPD

Zeocin

Baffled flasks

Procedure

1. *P. pastoris* from frozen glycerol stock was inoculated at 30℃ with shaking at 225 rpm, in 10 mL YPD with 100 µg/mL zeocin overnight.
2. The next day, the overnight culture was diluted to an OD_{600} of 0,15 – 0,2 in 50 mL YPD with 100 µg/mL zeocin and incubated in 30 ℃ for 2 – 3 days with shaking at 225 rpm.
    a. This volume can be scaled to higher ones.
3. The yeast cells were harvested by centrifugation at 2000 x g for 8 minutes. The supernatant was removed.
4. Cell pellet was resuspended in 5 mL dH_2O and centrifuged by 2000 x g for 8 minutes.
5. The cell pellet was stored at -20 ℃.

2.13.2 *L. plantarum* cultivation and harvesting

Materials

MRS

Erythromycin

Inducer pheromone (SppIP) (CASLO)

PBS

Procedure

1. *L. plantarum* was cultivated in MRS media with 10 µg/mL erythromycin at 37 ℃.
2. The overnight culture was diluted in 50 mL preheated MRS with 10 µg/mL erythromycin to a OD_{600} of 0,1 – 0,15 and incubated at 37 ℃.
3. When the incubated culture reached an OD_{600} of 0,3 ± 0,3, it was induced with 25 ng/mL SppIP and incubated for 3 hours at 37 ℃.
4. The bacteria cells were then harvested by centrifuge at 4250 x g for 7 minutes at 4 ℃.
5. The pellet was resuspended with 10 mL cold 1x PBS 3 times and centrifuged for 10 minutes at 4250 x g at 4 ℃ between each resuspension.
6. The pellet was resuspended in 1 mL PBS or stored at -20 ℃.

2.13.3 Cell disruption preparation

The harvested cells of *L. plantarum* were all treated the same before the following three sub-chapters.

Materials

Lysozyme

Phenylmethylsulfonyl fluoride solution (PMFS)

DNase

PBS

Procedure

1. The cell pellet was thawed and resuspended in PBS.
2. 2 µL PMFS per mL solution was added to the resuspended cells.
3. 1 µL DNase per 10 mL solution was added and mixed.
4. Lysozyme was added to the solution to the concentration of 0,1 – 1,0 mg/mL and incubated at 37 °C for 1 hour.

2.13.3.1 High-Pressure Homogenizers - Microfluidizer® processor

The Microfluidizer® uses high pressure to shear cells, pushing a single cell through a tiny tube that gradually becomes smaller until the cell wall breaks down. When cells are disrupted using this method, large fragments of the cell wall remain from the shearing.

Materials

Schott Duran bottle

dH_2O

LM20 Microfluidizer

Lysis buffer (20 mM TrisHCl pH 7)

70 % ethanol

Treated cell-suspension

Procedure

1. Ice, Duran bottles with: Lysis buffer, dH_2O and an empty one for waste, was prepared and brought to the LM20 Microfluidizer machine.
2. The tray beneath the cooling coil was filled with ice so the coil was completely covered and re-filled during the runtime.

3. The machine used the parameter 30.000 PSI for *L. plantarum*.
4. 100-200 mL dH_2O was poured into the reservoir at a time for washing, and air bubbles was removed with the stick accompanying the machine. The motor was then turned on.
5. Washing with water was repeated until 1 L dH_2O total had been used.
6. 100 mL Lysis buffer was poured into the reservoir.
7. After Lysis buffer had run through, the cell suspension was added to the reservoir and collected into an empty Duran bottle.
8. After lysis, dH_2O was added to wash the system until the waste became clear.
9. The system was cleaned and filled with 70 % ethanol for storage.
10. The motor was then stopped, and the machine turned off.
11. The protein extract was kept on ice, and then centrifuged at 18.000 x g for 10 minutes.
12. The sample was then kept on ice for purification later the same day.

2.13.3.2 Sonication

Sonication refers to the act of applying sound energy to agitate and shear cells. In this study, ultrasonic frequencies (>20 kHz) are applied to bacteria cells in to break and disrupt their cell walls and release the cellular contents, with the aim to extract proteins.

Materials

Treated cell suspension

Ice

Ultrasonic probe

Procedure

1. Treated cell suspension was placed on ice and put into the chamber.
2. The ultrasonic probe was placed 1-2 cm into the suspension without touching the walls of the container.

3. The ultrasonic probe used the following parameters:

   Runtime: 3 seconds

   Pause: 2 seconds

   Time 10 minutes x 3

   Frequency: 25 kHz

4. The container was adjusted during the runtime to avoid the probe hitting the walls of the container, and ice was re-filled when necessary.
5. After lysis, the protein extract was centrifuged at 18.000 x g for 10 minutes and stored on ice for purification later the same day.

2.13.3.3 FastPrep® 24 – Glass Beads

Materials

FastPrep® tube (Fisher Scientific)

FastPrep® - 24 Tissues and Cell homogenizer – Classic (MP Biomedicals)

Glass beads (Sigma)

PBS

Procedure

1. Harvested cell pellet was resuspended in 1 mL 1x PBS and transferred to a FastPrep® tube.
2. The tube was placed in the FastPrep® - 24 Tissues and Cell homogenizer machine and ran the following program: 6,5 m/s for 45 seconds and placed on ice for 5 minutes. This was repeated 1-3 times.
3. To gather the glass beads, the tube was centrifuged at 16.100 x g for 1 minute at 4 ℃ and the supernatant was transferred to a new Eppendorf tube.
4. The Eppendorf tube was centrifuged again at 16.100 x g for 2 minutes at 4 ℃.
5. Steps 3 and 4 were repeated until there was no sign of a pellet.

6. The protein extract was stored at -20 ℃.

2.14 Western blot

Western blot is an antibody-hybridization technique to detect specific proteins in a sample. The sample is first run through gel electrophoresis using SDS-PAGE, where proteins are separated by size, and then transferred to a membrane through electroblotting. Bovine serum albumin (BSA) is added to a blocking solution which the membrane is then exposed for, hindering un-specific binding of antibody and the protein. After the blocking step is complete, the primary antibody is applied to the membrane which binds to the target protein. The membrane is then exposed for the secondary antibody, which binds to the primary antibody. The secondary antibody is conjugated to horseradish peroxidase (HRP), which produces light-signals when cleaving substrates such as luminol, which is chemiluminescent.

2.14.1 Blotting with iBlot® Dry Blotting System

Materials

iBlot® Gel Transfer Stack

iBlot® Dry Blotting System

Pre-run gel

iBlot® Disposable Sponge

Blotting Roller

Nitrocellulose membrane

Procedure

1. Pre-run gel was removed from gel cassette and briefly placed in dH_2O to wash away gel fragments.
2. The iBlot® Gel Transfer Stack was opened, and the iBlot® Anode Stack, Bottom was removed from the package and placed in the blotting surface, with the plastic container attached.
3. The gel was placed onto the iBlot® Anode Stack. The membrane was briefly soaked in dH2O before the it was aligned on top of the gel again.
4. The Blotting Roller was gently used to remove any air-bubbles between the membrane and gel.
5. The iBlot® Cathode Stack was placed on top of the membrane, and the Blotting Roller was again gently used.
6. The iBlot® Disposable Sponge was placed on the inner lid of the iBlot® Dry Transfer System machine, with the metal contact on the sponge to the right corner of the sponge holder.
7. The lid was closed and secured. The program used was loaded onto the machine (P3).
8. After completed run, the stack was opened, and the membrane placed in dH_2O before further use.

2.14.1.1 Blotting with Tank transfer system Mini Trans-Blot cell

Blotting with the Tank transfer system was used in this study due to a lack of materials to perform the blotting using the iBlot Dry Blotting System.

Materials

Mini Trans-Blot cell

Buffer tank

Gel holder cassette

Bio-Ice® cooling unit

Fiber pads

Filter paper

Nitrocellulose membrane

Towbin buffer

Pre-run gel

Solutions

Towbin buffer

25 mM Tris-base

192 mM glycine

20 % methanol

Procedure

1. Pre-run gel was washed in dH_2O for 15 minutes, and the membrane was briefly soaked in methanol.
2. The membrane and gel were equilibrated in Towbin buffer for 15 minutes.
3. Filter paper and fiber pads was briefly soaked in Towbin buffer, before the blotting sandwich was assembled (Figure 2.1).
4. The sandwich was placed in the holder and put into the buffer tank along with the pre-cooled cooling unit (at -80 ℃).
5. Towbin buffer was poured into the buffer tank up to the tank rim.
6. The tank was placed under magnet stirring for 1 hour while the protein transfer took place, under 100 V.

7. The blotting sandwich was then opened, and the membrane was ready for hybridization.

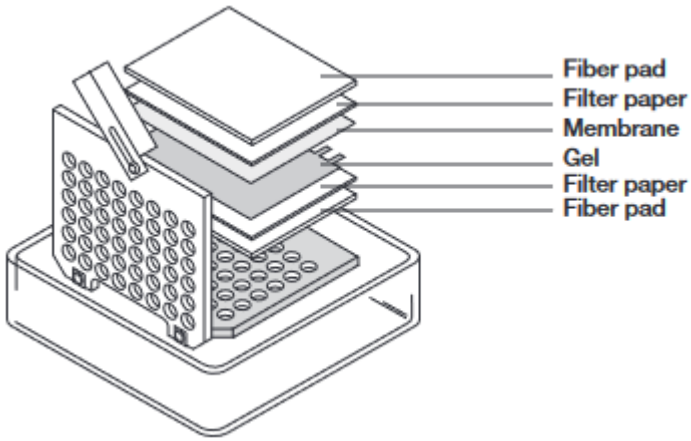


Figure 2.1. Assembly of blotting sandwich for Mini-Trans-Blot Cell. Taken from iBlot® Dry Blotting System Quick Reference by Invitrogen.

2.14.2 SNAP i.d.® immunodetection

To hybridize antibodies to the proteins on the membrane, the SNAP i.d. ® immunodetection system is used. This system lets the different solutions (blocking, washing and antibodies) run through the membrane using vacuum, which reduces the time required for hybridization compared to traditional methods.

Materials

SNAP i.d. ® Protein Detection System

TTBS

TBS (Tris-Buffered Saline)

Blocking solution

Primary antibody

SARS-CoV-2-Spike-RBD Polyclonal Antibody (MyBioSource)

Penta-His Antibody, BSA Free

Secondary antibody

HRP-Mouse Anti-Rabbit IgG (Invitrogen)

HRP-Rabbit Anti-Mouse IgG (A9917) (Invitrogen)

BSA

Solutions

1 L TBS:

24 g Tris Base (Sigma)

88 g NaCl

Up to 1 L dH_2O

pH adjusted to 7,6

TTBS:

TBS

0,1 % w/v tween-20 (Sigma-Aldrich)

Blocking solution:

TTBS

3 % BSA

Procedure

1. Blot holder was wetted with dH_2O and the membrane (2.14.1.) was placed in the holder with the protein side down.

2. Blot holder was placed in the SNAP i.d. ® system with the protein side up.
3. 30 mL blocking solution was poured into the container and the vacuum was turned on. 10 mL blocking solution was added at a time.
4. The vacuum was running until the blocking solution was completely gone, before being shut off.
5. The primary antibody was diluted in 5 mL blocking solution, gently mixed, and poured over the container and incubated for 10 minutes.
    a. For detection of SARS-CoV-2-Spike-RBD, 5 µL antibody was added.
    b. For detection of His-tag, 2 µL antibody was added.
6. The vacuum was turned on, and the membrane was washed three times with 30 mL TTBS until all liquid had run through before being shut off again.
7. The secondary antibody was diluted in 5 mL blocking solution, gently mixed and poured over the container and incubated for 10 minutes.
    a. For detection of SARS-CoV-2-Spike-antibodies, 0,25 µL HRP-Anti-Rabbit was added.
    b. For detection of His-tag, 0,4 µL of HRP-Rabbit Anti-Mouse was added.
8. The vacuum was turned on, the membrane washed with 30 mL TTBS three times, and the vacuum was turned off again.
9. The membrane was removed from the blot holder.

2.14.3 Chemiluminescence detection of proteins

Materials

SuperSignal ® West Pico Chemiluminescent Substrate

Luminol/Enhancer

Stable Peroxide Buffer

Pre-run Membrane

Procedure

1. 5 mL of Stable Peroxide Buffer and 5 mL of Luminol/Enhancer was mixed into a solution.
2. The pre-run membrane (from 2.14.2 and 2.14.3) was placed in an empty container, and incubated in the solution, free from light exposer, for 5 minutes.
3. The solution was poured away, and the membrane was ready for imaging by the Azure c400 machine.

2.15 Protein Purification

2.15.1 Akta Pure Protein Purification System

To purify His-tagged proteins in this study, Akta Pure chromatography was used. It is a system that allows for easy protein purification.

Materials

Buffer A

20 mM TrisHCl pH 8

Buffer B

20 mM TrisHCl pH8

500 mM imidazole

Histidine column (5 mL volume)

Glass tubes

70 % ethanol (Sigma-Aldrich)

dH_2O

Protein extract sample

Procedure

1. The system was prepared by setting the start flow at 2,5 mL/min and running 25 mL 70 % ethanol through it. This was done to remove potential residues in the system. The system flow was set to run through the entire system, including the "column".
2. 25 mL dH_2O was then run through the system to remove the ethanol.
3. The system was set to pause, and the histidine column was attached, before start running another 25 mL dH_2O through the system.
4. The protein extract (from 2.13.3.1 – 2.13.3.3) was run through the system.
5. When all the protein extract was in the system, the buffers A and B was both attached to the system. The ratio of the buffers was set to start at 100:0, and gradually changing over 50 minutes to 0:100, letting 100 mL total buffer run through the column with 2,0 mL/min flow. The out-flow of the system was then fractionated in glass tubes.
6. After purification, the fractionation stopped, and the system was cleaned by running dH_2O through it first, before ending with 70 % ethanol.
7. The collected outflow was stored in a refrigerator at 4 ℃.

2.15.1.2 Buffer shift

After the protein purification procedure was over, it was necessary to change the solution or buffer that the proteins were contained in, to improve the stabilization of the proteins.

Materials

Sample

PBS

Protein collection tube

Procedure

1. 15 mL sample was transferred to a protein collection tube and centrifuged at 4250 x g for 20-40 minutes.

2. The bottom waste was thrown away, and 15 mL PBS was added to the collection tube. The tube was then centrifuged at 4250 x g for 20-40 minutes.
3. Step 2 was repeated for a total of 5 times.
4. The upper contents of the collection tube were then transferred to another container and stored at 4 ℃.

2.15.2 Ni-NTA Magnetic Agarose Beads

Ni-NTA Magnetic Agarose Beads are magnetic particles covered with an affinity purification matrix. These beads are used for purifying and immobilizing proteins with a His-tag. There are empty spots in the affinity purification matrix on the beads that bind histidine residues with high affinity, and once the protein is bound, the beads can be gathered using magnets.

Materials

Binding buffer

50 mM HEPES

20 mM Imidazole

200 mM KCl

10 % Glycerol

PBS

Elution buffer

50 mM HEPES

200 mM KCl

500 mM Imidazole

10 % glycerol

NiNTA beads

Magnet

Procedure

1. 500 mL Binding buffer was added to an Eppendorf tube with 100 µL NiNTA beads for equilibration. The tube was placed next to a magnet for 30 seconds, and the buffer was removed.
2. 500 µL of protein extract (from 2.13.3.3) was added to the tube with the NiNTA beads and incubated at room temperature for 1 hour in a rotating stand.
3. The Eppendorf tube was then held next to a magnet for 30 seconds, and the protein extract was removed.
4. 500 µL washing buffer was then added to the tube and vortexed well, before placed next to the magnet for 30 seconds again. The washing buffer was removed.
5. Step 4 was repeated.
6. 30 µL elution buffer was added to the tube, vortexed well, and incubated at room temperature for 1 minute. It was then placed next to the magnet for 30 seconds, before the elution was removed and stored at 4 ℃.
7. Step 6 was repeated.

2.16 Detecting surface localized proteins on *L. plantarum*

2.16.1 Binding of LysM-fused antigens to *L. plantarum*

Materials

Protein extract

Purified protein

PBS

Bacteria culture

MacsQuant® Analyser

MacsQuantify™ Software

Procedure

1. Bacteria cultures were incubated overnight according to their specific requirements.
    a. For example, *L. plantarum* was prepared the same way as described in section 2.13.2, steps 1 and 2.
2. A small sample was taken from the culture and analyzed by the MacsQuant® Analyser, counting the amount of culture needed for approximately 500.000 cells. This amount was then harvested from the culture by centrifuge with 5000 x g for 3 minutes, and the supernatant was removed.
3. The pellet was washed in 250 µL PBS and centrifuged with 5000 x g for 3 minutes. This was done two times.
4. The cell pellet was resuspended in 0,5 – 1,0 mL protein extract (section 2.13.3.1 – 2.13.3.3) or purified protein (section 2.15.1) and incubated in varying temperatures and times (Results section…).
5. The sample was centrifuged at 5000 x g for 3 minutes, and supernatant removed.
6. The cell pellet was washed in 250 µL PBS and centrifuged by 5000 x g for 3 minutes. The supernatant was removed. This was done three times.
7. The cell pellet was now ready for further analysis by Flow cytometry.

2.16.2 Flow cytometry analysis

To detect proteins localized on the surface of a bacteria, antibodies conjugated with fluorochromes can be used. A fluorochrome is a fluorescent compound that can absorb light at certain wave lengths and then emit a light signal at a higher wavelength. With the use of a primary antibody that binds to the target protein, and a secondary antibody that has an fluorochrome attached, which binds to the primary antibody, one can ascertain if

the target protein is exposed on the surface of the bacteria. The fluorochrome used in this study is Fluorescein Isothiocyanate (FITC).

Flow cytometry sends a single cell through a laser beam at a time. If the cell has proteins on the surface that has FITC attached, a light signal will be emitted, and can thus be analyzed.

Materials

BSA

PBS

MacsQuant® Analyser

MacsQuantify™ Software

Primary antibody

SARS-CoV-2-Spike-RBD Polyclonal Antibody

Penta-His™ Antibody, BSA Free

Secondary antibody

Anti-Mouse IgG-FITC (F0257)

Anti-Rabbit IgG-FITC (F9887)

Procedure

1. *L. plantarum* cells were prepared as described in section 2.16.1.
    a. Or, based on OD_{600} values measured of the culture, $\frac{500}{OD600}$ µL was harvested. If the OD_{600} value was 1, 500 µL were harvested, if it was 2, 250 µL was harvested.
2. The cells were centrifuged at 500 x g for 3 minutes, and supernatant removed.
3. Cell pellet was washed with 1 mL PBS and centrifuged at 5000 x g for 3 minutes, and supernatant removed.

4. Cell pellet was resuspended in 50 µL PBS + 2% BSA and 0,2 µL primary antibody and incubated at room temperature for 30 minutes.
    a. For detection of SARS-CoV-2-RBD, SARS-CoV-2-Spike-RBD Polyclonal Antibody was used.
    b. For detection of His-tag, Penta-His™ Antibody, BSA Free was used.
5. Sample was centrifuged at 5000 x g for 1 minute, and supernatant was removed by pipette.
6. Sample was washed with 600 µL PBS + 2 % BSA and centrifuged at 5000 x g for 2 minutes.
7. Step 6 was repeated 2 more times.
8. Cell pellet was resuspended in 50 µL PBS + 2 % BSA + 0,3 µL secondary antibody containing FITC, and incubated for 30 minutes in room temperature, shielded from light.
9. Step 5 was repeated.
10. Step 6 was repeated 4 times.
11. The cell pellet was resuspended in 1 mL PBS.
12. For preparation of analysis, 100 µL of sample was added to 900 µL PBS.

3 Results

The results of this study are introduced in the following chapter. A total of four vectors containing SARS-CoV-2 antigens were constructed. The pOST-1 expression system was used in the construction of the two plasmids in *Pichia pastoris*, and the pSIP system was used in the construction of the two plasmids in *Lactiplantibacillus plantarum.* All vectors were constructed in *E. coli*, then transformed into *P. pastoris* or *L. plantarum*. Protein precipitation, western blot analysis and protein purification was performed for both vectors in *P. pastoris*. Growth curve analysis, western blot analysis, protein purification and flow cytometry analysis were performed on the recombinant *L. plantarum*. Protein precipitation was performed to concentrate and purify proteins in *P. pastoris* supernatants for further analysis. Western blot analysis was carried out for all four constructs to study

the production of antigens in *P. pastoris* and *L. plantarum*. Growth curve analysis was done to study the effect of the rate of growth on recombinant *L. plantarum* induced with the pheromone SppIP. Flow cytometry analysis was used to detect surface binding of the antigens on the recombinant *L. plantarum*. The results for the constructs in *P. pastoris* and *L. plantarum* are separated into different sections.

3.1 SARS-CoV-2 constructs

Plasmids containing LysM-fused SARS-CoV-2 antigens were constructed in this study. The plasmids contained a subunit of the spike protein from SARS-CoV-2, the receptor-binding domain (RBD). The spike protein of the SARS-CoV-2 virus is essential for it to bind and infiltrate host cells, and replicate. It is for this reason that the RBD subunit is chosen to be expressed by these constructs.

The two plasmid constructs in *P. pastoris* both contain the RBD subunit. One of the plasmids were constructed with a single chain dimer (sc) of RBD, as Dai et al. (2020) showed that the sc_RBD construct induced a better immune response than a monomer of RBD. Both plasmid constructs for *L. plantarum* contained a monomer of RBD. One of the plasmids were constructed without a signal sequence for transportation of the protein through to the cell wall, with a histidine tag attached to enable protein purification later in the study.

3.1.1 Construction of SARS-CoV-2 antigen vectors in *Pichia pastoris*

For the construction of the LysM-fused SARS-CoV-2-RBD antigen in *P. pastoris*, the LysM domain from the Lp_3014 protein in *L. plantarum* WCFS1 was utilized. For both constructs, the plasmid pBSYGCW$_{14}$Z was used as the basis, as it contained “Gibson overhangs” on both sides of a “stuffer fragment”. The stuffer fragment would be removed by the restriction enzyme digestion of SapI, generating overlapping DNA fragments (Gibson overhangs).

The LysM anchored monomer RBD plasmid pBSYGCW$_{14}$Z -Ost-1_LysM_RBD_DC (LysM_RBD_DC) was constructed by digesting the 3717 bp pBSYGCW$_{14}$Z-Ost-1 plasmid with SapI, removing the 442 bp stuffer fragment (Figure 3.1). The constructed

1093 bp LysM_RBD_DC_His fragment replaced the stuffer fragment through Gibson Assembly ligation, yielding the 4313 bp LysM_RBD_DC plasmid.

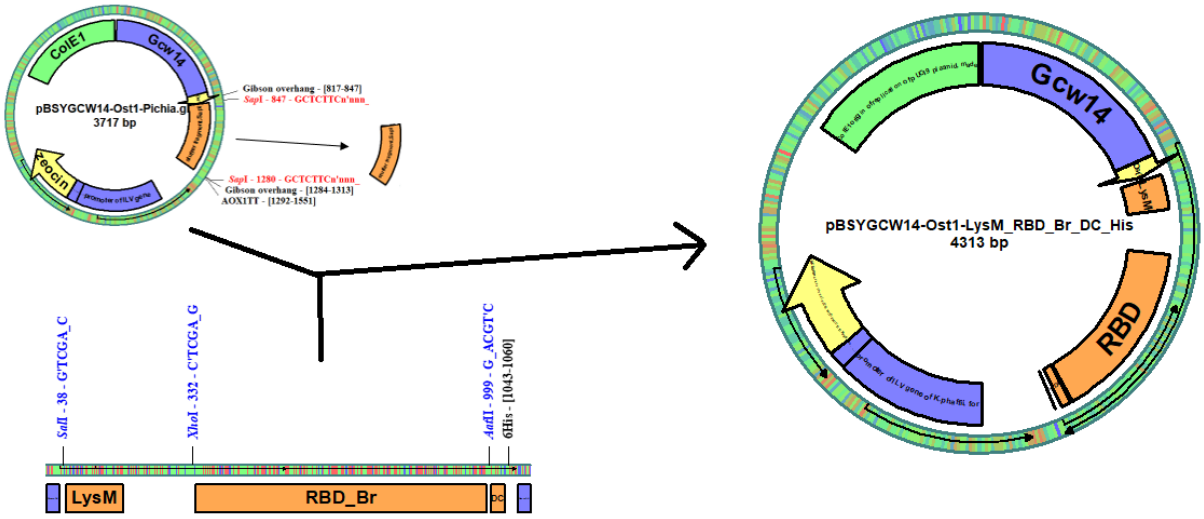


Figure 3.1. Strategy for constructing the pBSYGGCW$_{14}$Z-ost1-LysM_RBD_DC_His vector.

The LysM_sc_RBD plasmid (pBSYGGCW14Z-ost1-LysM_scRBD_DC_His) was constructed by digesting the 3717 bp pBSYGCW$_{14}$Z-Ost-1 plasmid with SapI, removing the 442 bp stuffer fragment, and the 4124 bp pMT-A-3014_scRBD_DC_6His with SalI (Figure 3.2). The 1750 bp LysM_scRBD_DC_His fragment replaced the stuffer fragment by Gibson Assembly ligation, yielding the 4970 bp pBSYGGCW14Z-ost1-LysM_scRBD_DC_His vector.

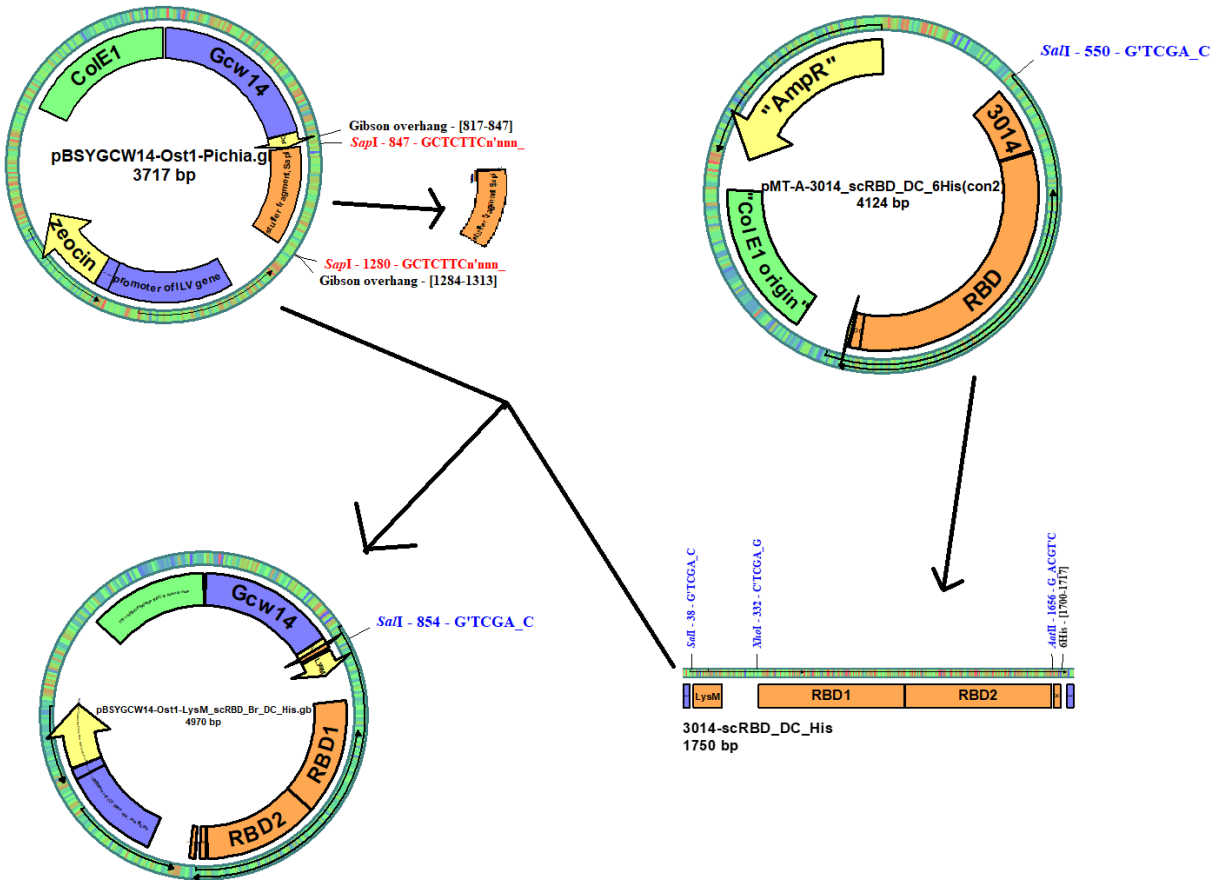


Figure 3.2. Strategy for construction of the pBSYGGCW14Z-ost1-LysM_scRBD_DC_His vector.

3.1.2 Expression of LysM-fused SARS-CoV-2-RBD antigen in *P. pastoris*

P. pastoris was used to express two the LysM-fused SARS-CoV-2-RBD antigens, “RBD” and “sc_RBD”. Freshly made electrocompetent *P. pastoris* cells were transformed with the two constructed vectors, as transformation with *P. pastoris* needs to be done with cells made competent the same day. After 3 – 5 days, the grown colonies were sampled for long-term storage, and inoculated in 25 mL of YPD with antibiotics for 2 – 3 days because of slow growth. This was repeated twice, as the first transformation gave no positive transformations for both constructs.

To confirm that the proteins were produced, supernatants after centrifugation of the inoculated cultures were run through an SDS-PAGE. A total of 66 colonies were tested: 28 colonies producing LysM_RBD and 38 colonies producing LysM_scRBD. Supernatants run directly on the gel produced only smears when visualized after gel electrophorese (not shown). Optimalizations then led to methanol-chloroform protein precipitation. When this procedure was done on the supernatants before being run on an SDS-PAGE gel, bonds would be visible (Figure 3.3). The expected theoretical molecular weight of the LysM_RBD protein was 40,5 kDa, and 65 kDa for LysM_scRBD. No clear bonds were visible after all 66 colonies were tested.

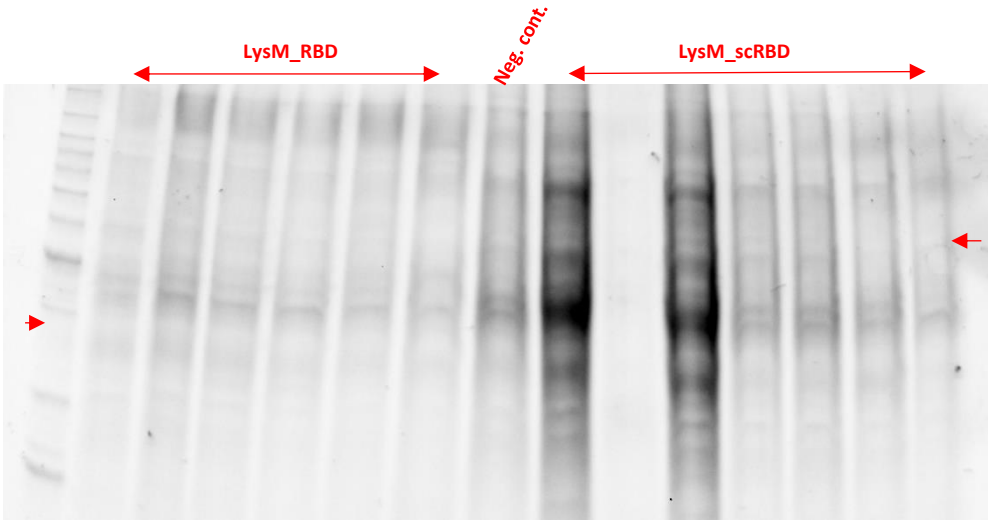


Figure 3.3. Representative SDS-PAGE gel of *P. pastoris* harboring the constructed plasmids (LysM_RBD & LysM_scRBD) after methanol-chloroform protein precipitation. Left of negative control (Neg.cont.): LysM_RBD (40,5 kDa). To the right of negative control, LysM_scRBD (65 kDa).

Certain colonies that showed possible bonds were chosen for further study after many SDS-PAGE runs with protein precipitation (not shown). Multiple colonies harboring either construct was grown overnight in YPD medium and antibiotics and harvested by centrifugation. The cell pellets were washed and resuspended in PBS, before being disrupted with glass beads in a FastPrep-24 machine. The protein extract was then run on an SDS-PAGE gel (Figure 3.4). This process was repeated multiple times as there were no clearly observed bonds that corresponded to the expected molecular weights of

LysM_RBD (40,5 kDa) and LysM_scRBD (65 kDa). Figure 3.4 shows a representative result of the many SDS-PAGE gels that were run.

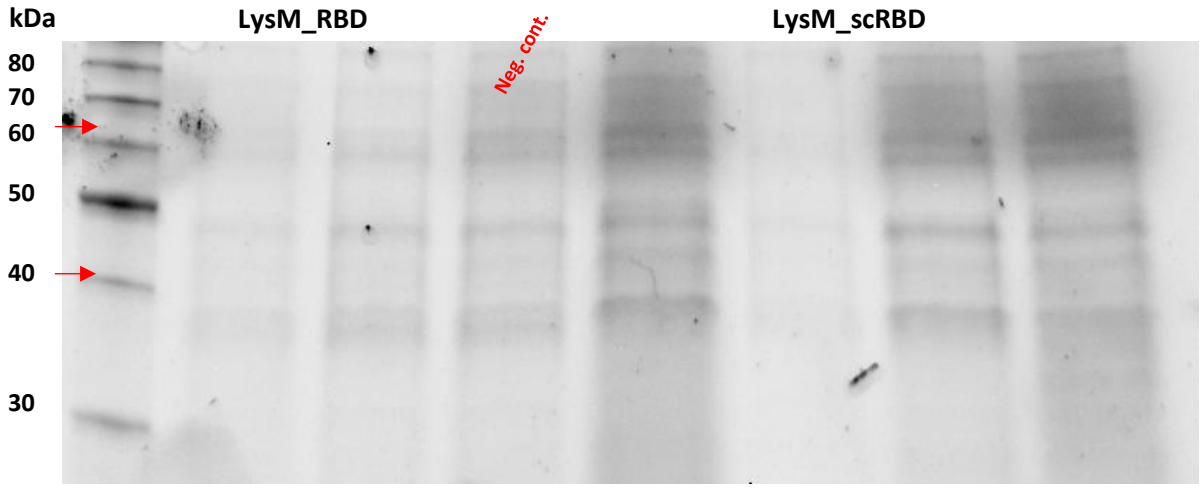


Figure 3.4. Representative SDS-PAGE of protein extract from disrupted *P. pastoris* cells harboring LysM_RBD and LysM_scRBD.

Since SDS-PAGE of supernatant and protein extract of disrupted cells did not show any clear results, western blot analysis was performed. A few colonies that showed possible bonds (Figure 3.3 and 3.4) were chosen for this analysis. *P. pastoris* cells were harvested from cultures inoculated for 2 – 3 days, washed and resuspended in PBS, and disrupted with glass beads. The amount of culture disrupted were equilibrated based on the lowest OD_{595} measured before harvesting. The protein extract was run on an SDS-PAGE gel before being transferred to a nitrocellulose membrane. Antigens were hybridized with specific antigens before being visualized with chemiluminescence (Figure 3.5). This procedure was repeated multiple times with varying results due to optimizations (amount of culture disrupted and run on SDS-PAGE, amount of antibodies added for hybridization), with the most interesting result presented in Figure 3.5.

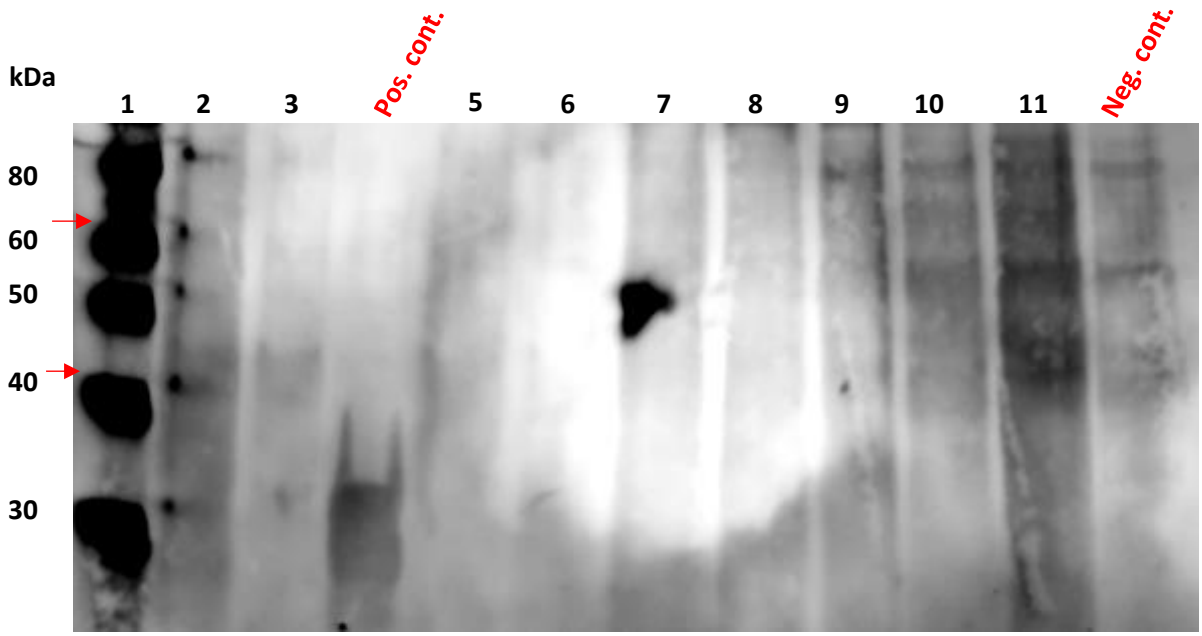


Figure 3.5. Western blot of SARS-CoV-2-RBD antigens (LysM_RBD & LysM_scRBD). Well 1: Magic Marker XP. Well 2 & 3: LysM_RBD. (40,5 kDa) Well 4: Positive control. Well 5-11: LysM_scRBD (65 kDa). Well 12: Negative control.

Figure 3.5 shows the results of the western blot analysis. There are some observed bonds that can correspond to the expected molecular weight of the proteins. In well 3 the bond length is a little higher than expected (40,5 kDa), and there is an observable bond in well 10that is also a little higher than the expected 65 kDa weight of LysM_scRBD.

3.1.3 Purification of SARS-CoV-2-RBD antigens from protein extracts of *P. pastoris*

The same cultures used for western blot analysis in Figure 3.5 were chosen for protein purification. *P. pastoris* cultures were grown for 2-3 days and harvested by centrifugation, before the cell pellet was washed and resuspended in PBS and disrupted with glass beads. The protein extract was cleaned of glass beads by centrifugation and washing with PBS, before being resuspended in a buffer A (Section 2.15.2) prepared for the purification process. Supernatant from grown cultures was also tested. Since the SARS-CoV-2-RBD antigens contain a Histidine-tag, purification of the protein with Ni-NTA magnetic agarose beads was performed. Buffer A would contain a low concentration of imidazole (20 mM) to prevent unspecific interactions, whereas Buffer B contained a high concentration (500 mM) of imidazole to ensure the release of the bound protein. Eluate

from the purification process was run on an SDS-PAGE before a western blot analysis were performed. This procedure was repeated multiple with the same Ni-NTA agarose beads for each sample due to bad results (Figure 3.6).

There are no detectable SARS-CoV-2-RBD in any eluates in Figure 3.6. A Bradford Assay of the eluates was then performed, where none of the samples had a protein concentration high enough to be measured by the method. Repeated attempts with using varying concentrations of imidazole in Buffers A (0 mM), amount of buffer added to the protein extract during purification or elucidation, gave the same results.

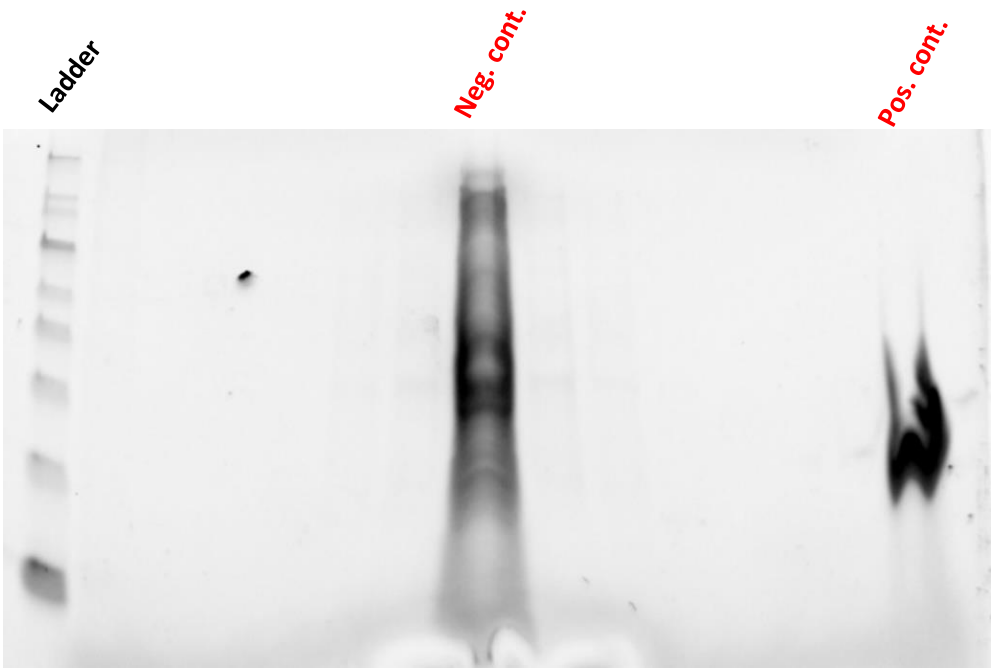


Figure 3.6. Western blot of purification process of His-tag SARS-CoV-2-RBD antigens. Eluate of protein purification using Ni-NTA magnetic agarose beads, with both negative and positive control. A representative result of the various times the procedure was done.

3.2.1 Construction of SARS-CoV-2 antigen vectors in *Lactiplantibacillus plantarum*

For the construction of the LysM-fused SARS-CoV-2-RBD antigen in *L. plantarum*, the LysM domain from pLp_3014_4Ag85E6-DC was used. There were two constructs created for *L. plantarum*: one with a signal sequence (pLp3014_LysM_RBD_DC), and one without, instead having an attached histidine tag sequence (pSIP3014_LysM_RBD_DC_His).

For the construction of pLp3014_LysM_RBD_DC, the 6462 bp plasmid pLp1261_NTD_RBD_DC (pLp1261_RBD) was digested using the restriction enzymes SalI/HindIII, freeing the 642 bp RBD fragment (Figure 3.7). The plasmid pLp3014_4Ag85E6-DC was digested using restriction enzymes SalI/HindIII to remove the 1194 bp 4Ag85E6 fragment, which is a Tuberculosis (TB) antigen. The RBD block was then added to the cut pLp3014_4Ag85E5 plasmid using Quick Ligase, yielding the 6834 bp pLp3014_LysM_RBD_DC construct.

For the construction of the pSIP3014_LysM_RBD_DC_His plasmid, the pLp3014_4Ag85E6 plasmid was digested with NdeI and HindIII to remove the bp 1808 TB antigen and LysM sequence block (Figure 3.8). The constructed pLp3014_LysM_RBD_DC plasmid was digested using NdeI and HindIII, separating the 1194 bp LysM + RBD fragment. This 1194 bp fragment was then amplified and cloned into the cut pLp3014_4Ag85E6 vector following the In-Fusion HD Cloning Kit protocol.

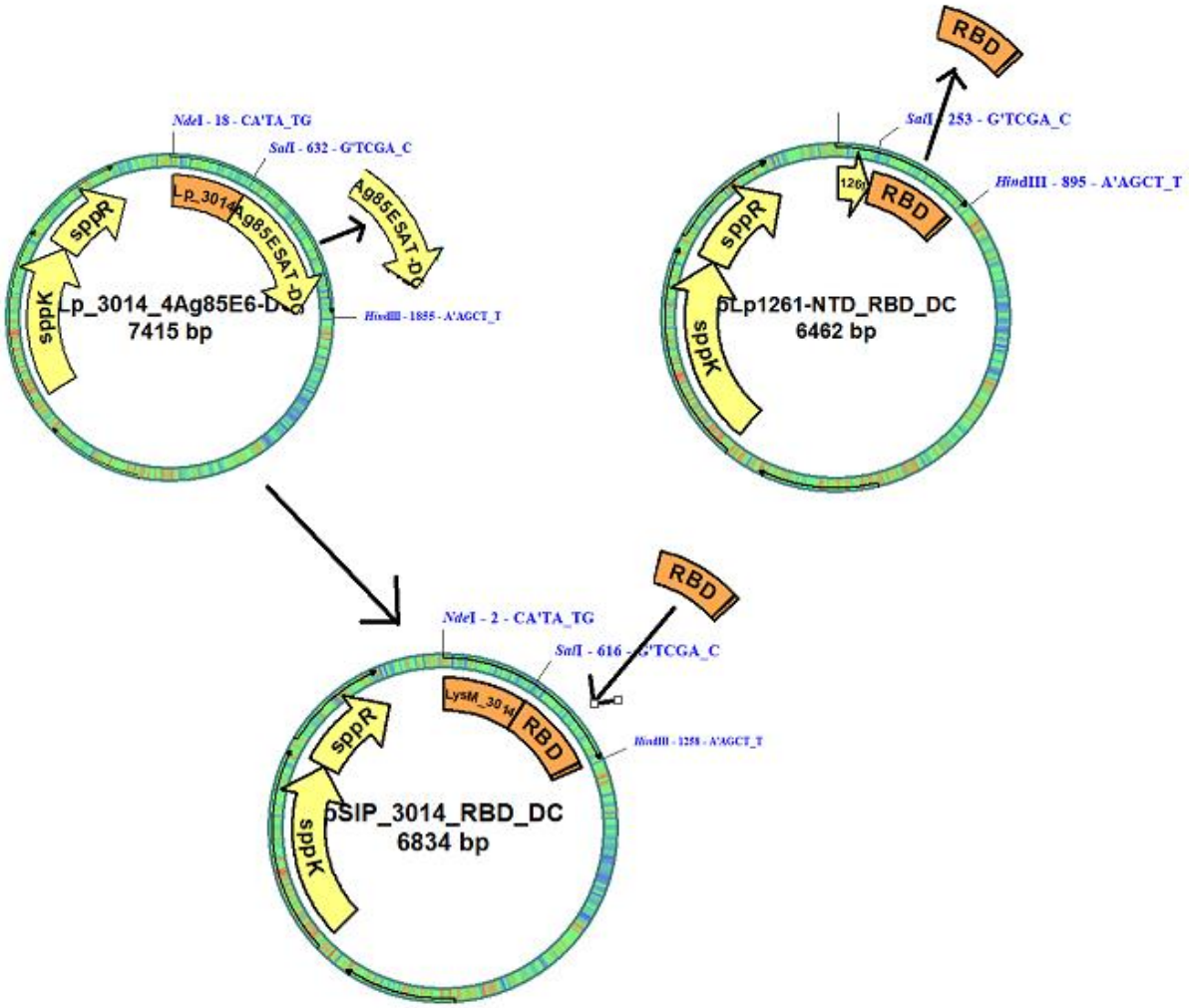


Figure 3.7. Strategy for constructing pLp_3014_LysM_RBD_DC vector.

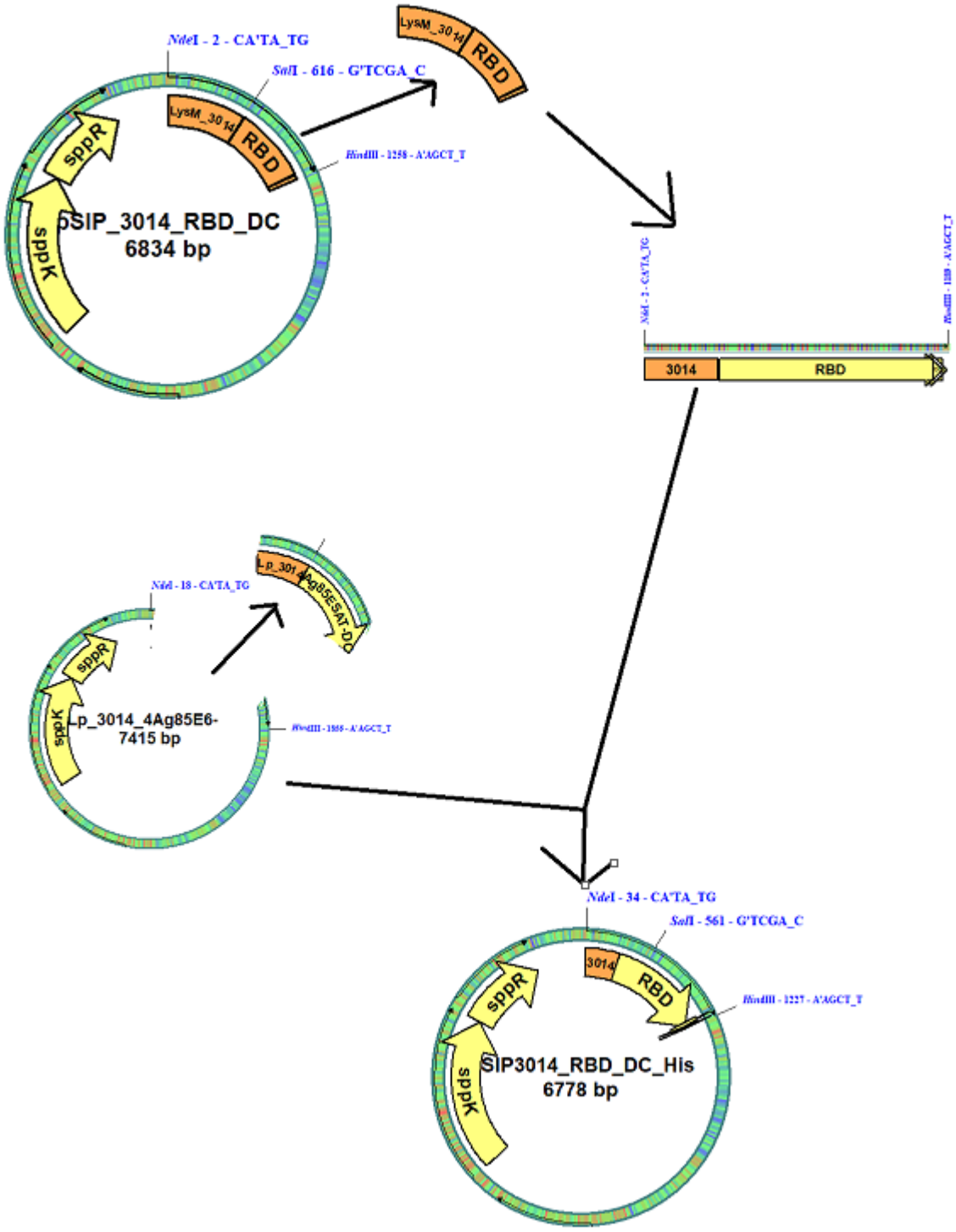


Figure 3.8. Strategy for constructing pSIP_3014_LysM_RBD_DC_His vector.

3.2.2 Growth curve analysis of *L. plantarum* harboring SARS-CoV-2 antigen vectors

To investigate the effect of expression of the SARS-CoV-2-RBD antigen on the growth rate of *L. plantarum*, a growth curve analysis was done. The growth curves of *L. plantarum* containing different SARS-CoV-2-RBD constructs are shown in Figure 3.9. 200 µL of both induced (SppIP 25 ng/mL) and non-induced bacteria cultures were added to a 96-well Microwell plate, and the growth rate was measured over 24 hours via OD_{600} every 5 minutes. Every sample was measured with triplicates, and the mean values was used in Figure 3.3. This growth curve analysis was done a total of two times with similar results.

L. plantarum harboring pEV (light blue) is included in the analysis for negative control (Figure 3.9). There are great differences between the uninduced and induced curves. The induced curves show significantly less growth than the uninduced curves, except for the pSIP_3014_LysM_RBD_DC_His, which maintains almost the same growth rate. Within the induced growth curves, there are significant differences between the curves. The constructed pLp_3014_LysM_RBD_DC has the least growth, with the pLp_3050_RBD (LPxTG anchor) having a struggling growth before barely overtaking the lipoprotein anchored pLp_1261_RBD in the last hour. The uninduced curves, pEV and WCFS1 strain shows similar growth curves, which is expected since there are no antigens expressed.

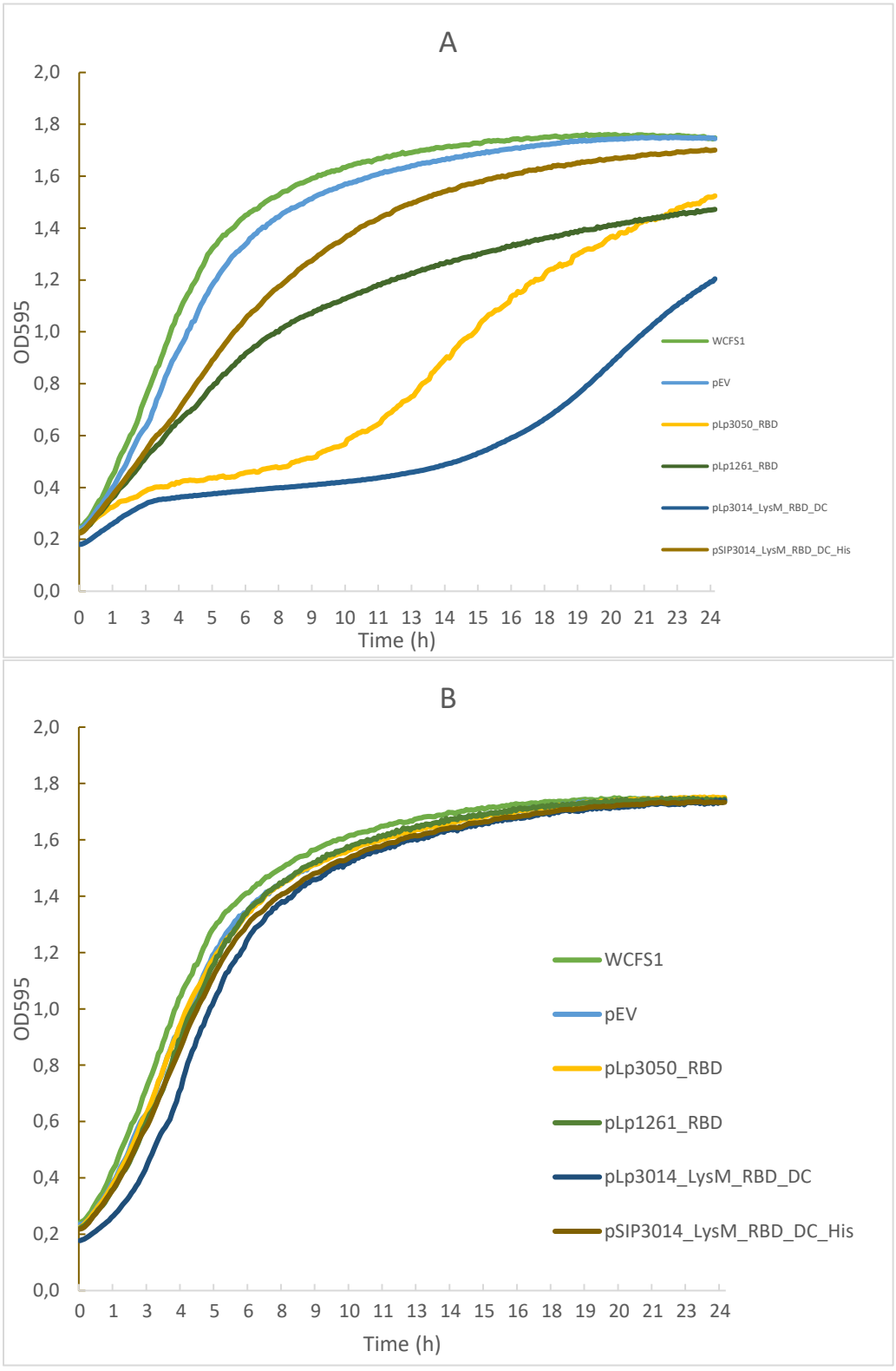


Figure 3.9. Growth curves of *L. plantarum* harboring different SARS-CoV-2-RBD constructs. A) Growth curves of induced bacteria, B) Growth curves of uninduced bacteria, The pEV plasmid and is included for negative control.

3.2.4 Detection of SARS-CoV-2-RBD antigens using Western blot

Western blot was used to analyze antigen production in *L. plantarum* harboring different SARS-CoV-2-RBD constructs. The bacteria containing the different constructs were harvested 3 hours after induction with 25 ng/mL SppIP and then disrupted for the proteins to be released form the cell interior. The protein extract was added to an SDS-PAGE gel before transferred to a membrane and blotted. The antigens were hybridized with specific antibodies using the SNAP i.d. immunodetection system and prepared for visualization with chemiluminescence.

Figure 3.10 shows strong bonds for all the different proteins, including the wells containing cell-pellets. Due to the poor visibility of the ladder (well 1), the bond lengths can only be estimated. The theoretical molecular weight of the proteins is 33 kDA (pLp_1261), 45 kDA (pLp_3014_LysM_RBD and pSIP_3014_LysM_RBD_His) and 47 kDA (pLp_3050_RBD). The pEV plasmid was included as a negative control and gave as expected no visible bonds.

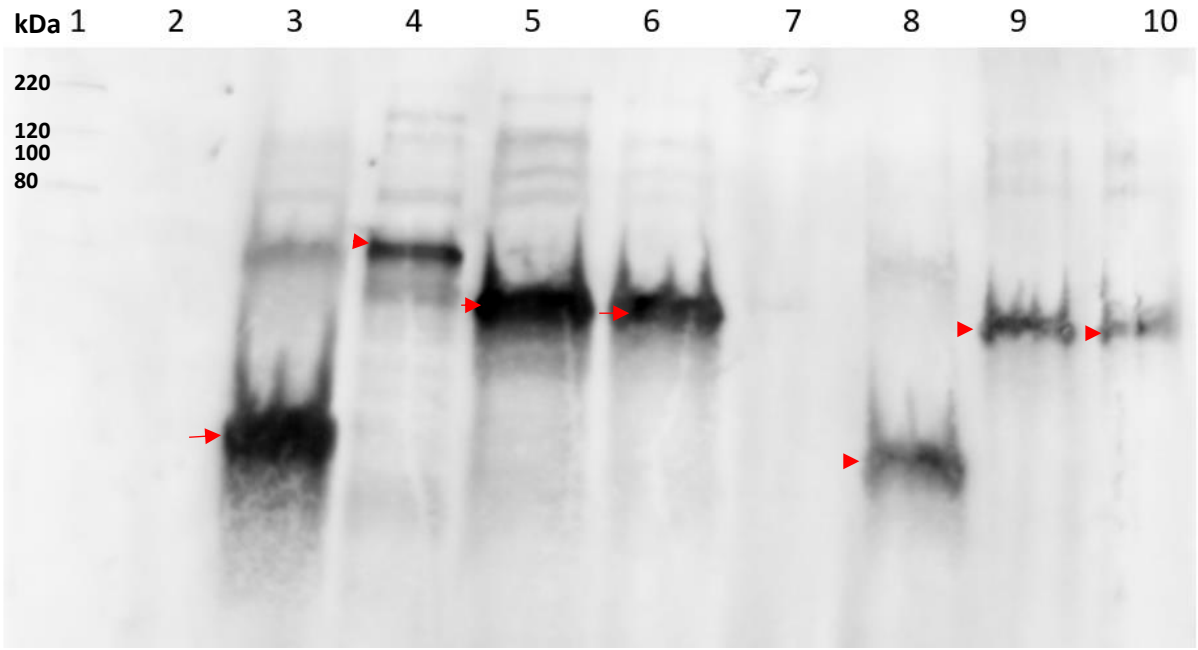


Figure 3.10. Western blot of *L. plantarum* harboring different SARS-CoV-2-RBD constructs.

Well 1: Magic Marker XP. Well 2: pEV. Well 3: pLp1261_RBD. Well 4: pLp3050_RBD. Well 5: pLp3014_LysM_RBD. Well 6: pSIP3014_LysM_RBD_His. Well 7: pEV cell-pellet. Well 8: pLp1261_RBD cell-pellet. Well 9: pLp3014_RBD cell-pellet. Well 10: pSIP3014_RBD cell-pellet.

3.2.5 Detection of SARS-CoV-2-RBD antigens using different lyse methods

Different methods of disrupting *L. plantarum* harboring SARS-CoV-2-RBD antigen was utilized in this study. This was done to find which method of disruption that resulted in the release of the most produced antigen in pSIP_3014_LysM_RBD_His.

3.2.5.1 Cell disruption by Microfluidizer

For cell lysing by Microfluidizer, *L. plantarum* harboring pSIP_3014_LysM_RBD_His was harvested and prepared as described in Section 2.13.3. After cell lysing, the protein extract was purified by Fast Protein Liquid Chromatography (FPLC). Western blot analysis was used to detect SARS-CoV-2 antigens in the protein purification eluate. The eluate was added to an SDS-PAGE gel before transferred to a membrane and blotted. The antigens were hybridized with specific antibodies using the SNAP i.d. immunodetection system and prepared for visualization with chemiluminescence.

Figure 3.11 shows the measured UV curve during the protein purification process. From the observed peaks, A and B are expected, as these comes after washing buffer is added. Peak C is extremely small and shows a steady growth, which is not expected. The expected peak should be sharp, signifying a mass release of the protein of interest, and not the flat C peak observed here.

Figure 3.12 shows weak bonds for all samples, except for well 8 with cell pellet from pSIP_3014_LysM_RBD_His. There is a slight bond from the lysate after cell shearing (well 3), and an even weaker bond in the eluate from the protein purification. The estimated theoretical molecular weight of the protein is 45 kDA, which fits the observed bonds. There are weaker bonds from pEV (well 2) which is included as a negative control, and the first wash (well 4).

A Bradford Assay was run to find the protein concentration in the eluate from the protein purification, but the protein concentration in the sample was too low for the Bradford Assay to estimate a value.

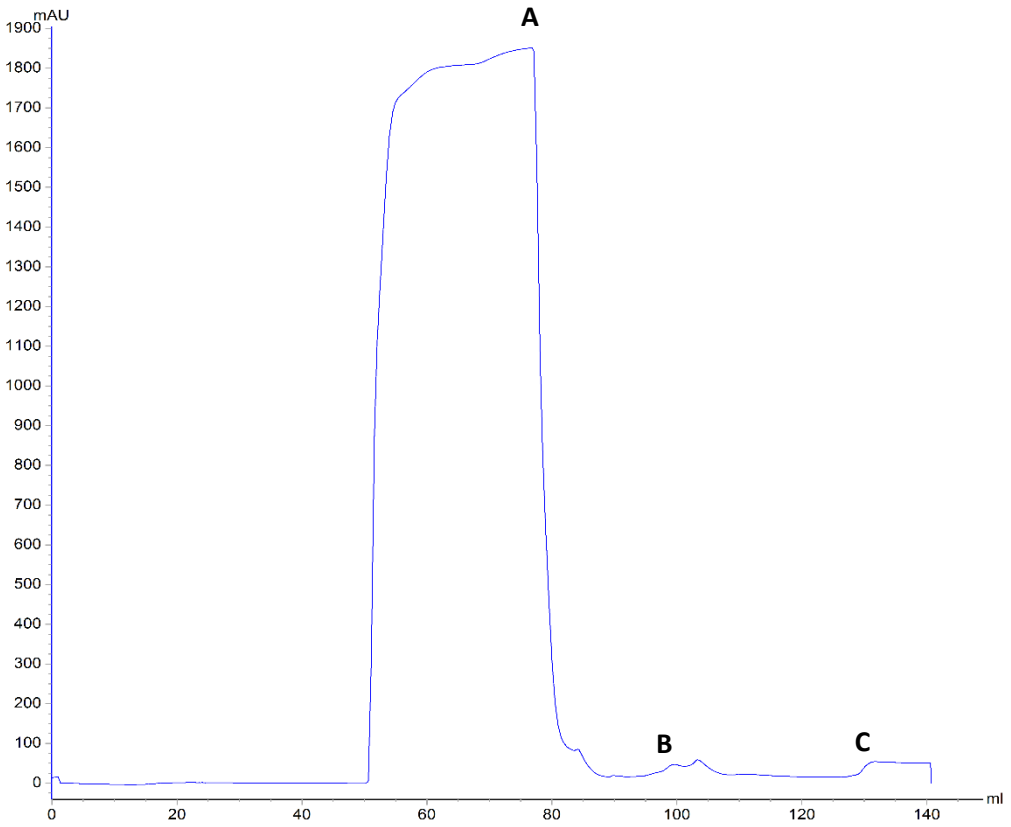


Figure 3.11. UV-curve from Fast Protein Liquid Chromatography from protein purification of protein extract. A: Washing buffer (A buffer) is added. B: Washing buffer (Buffer B) is added. C: Elution buffer is added.

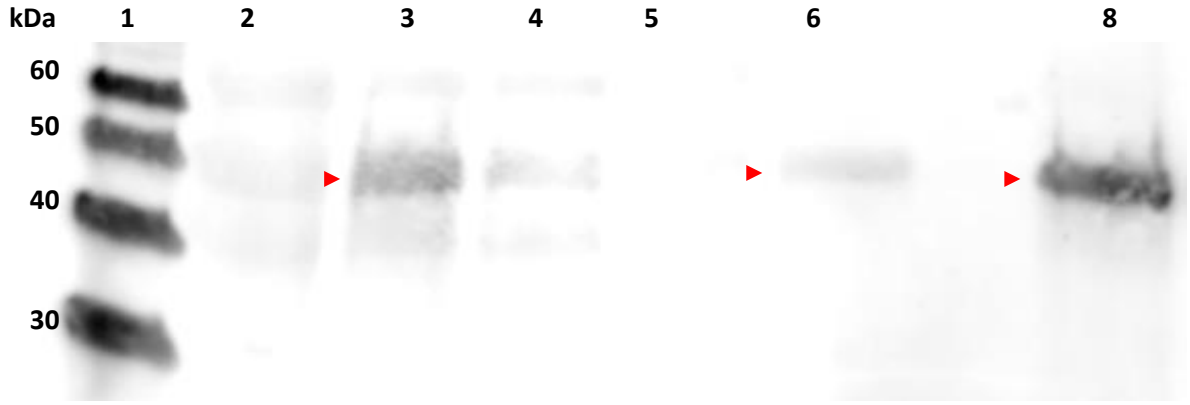


Figure 3.12. Western blot of protein purification from Microfluidizer protein extract of SARS-CoV-2-RBD antigens. Well 1: Magic Marker XP. Well 2: pEV. Well 3: Lysate after cell shearing. Well 4: Wash 1. Well 5: Wash 2. Well 6: Eluate. Well 8 pSIP_3014_LysM_RBD_His cell pellet.

3.2.5.2 Cell disruption by sonication

After the poor results of 3.2.5.1, cell disruption using sonication was explored. *L. plantarum* harboring pSIP_3014_LysM_RBD_His was harvested and prepared as described in Section 2.13.3. After cell lysis, the protein extract was purified by Fast Protein Liquid Chromatography (FPLC). The eluate from protein purification was concentrated in a Protein Concentrator tube by centrifugation with a filter of polyether sulfone (PES) and cellulose. Western blot analysis was used to detect SARS-CoV-2 antigens in the protein purification eluate. The eluate was added to an SDS-PAGE gel before transferred to a membrane and blotted. The antigens were hybridized with specific antibodies using the SNAP i.d. immunodetection system and prepared for visualization with chemiluminescence.

Figure 3.13 shows the UV curve from the protein purification via FPLC of the protein extract from sonication. There are two peaks observed, A and B. Peak A is expected, as this is when the washing buffer is added. Peak B has a short, sharp start, before gradually growing. This is more in line with what is expected to be observed during protein purification.

Figure 3.14 shows bonds from the lysate (well 3), concentrated eluate (well 6) and cell pellet of pSIP_3014_LysM_RBD_His, as well as in the pEV negative control. The theoretical molecular weight of the protein (psIP_3014_LysM_RBD_His) is 45 kDa, which matches the bonds in well 3, 6 and 8. The observed bonds are quite weak, but there is an obvious difference between the concentrated eluate with filters of PES (well 6) and cellulose (well 7).

A Bradford Assay was performed to find the protein concentration of the concentrated eluate. The protein concentration of the concentrated eluate with a PES filter had a protein concentration of 68,1 µg/mL, whereas the protein concentration in the concentrated eluate using a cellulose filter was too low to be measured.

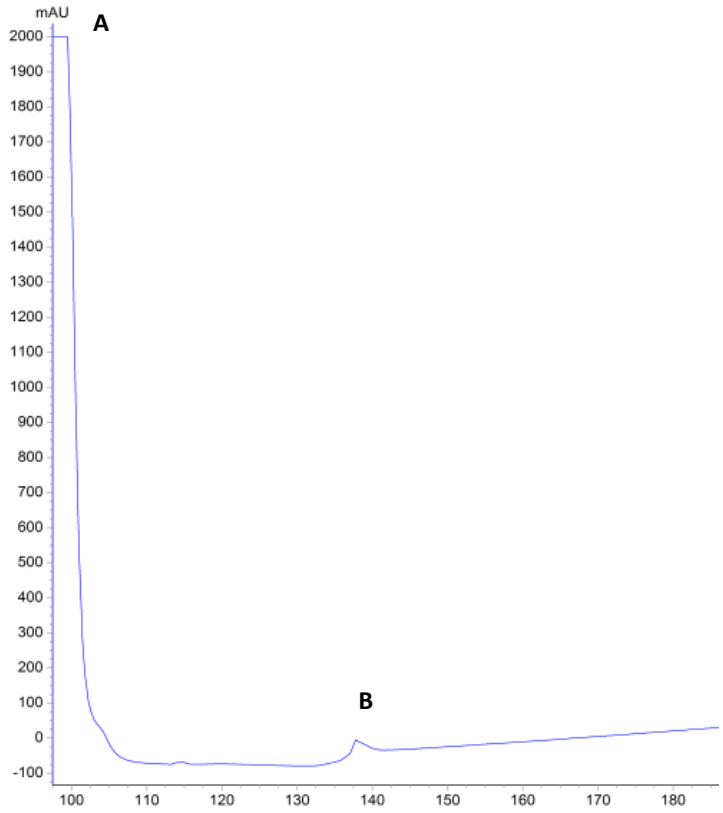


Figure 3.13. UV-curve from Fast Protein Liquid Chromatography from protein purification of protein extract.
A: Washing buffer (A buffer) is added. B: Elution buffer is added.

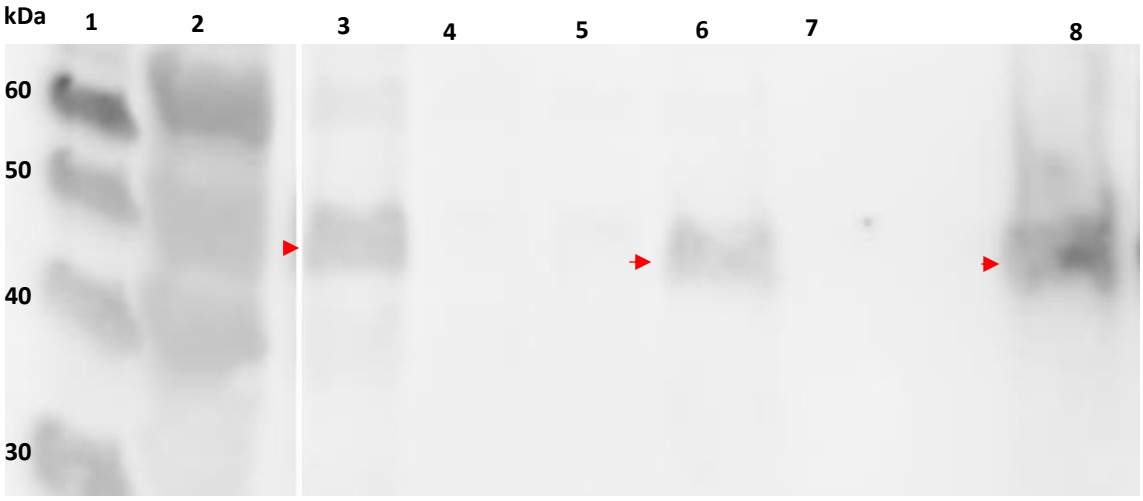


Figure 3.14. Western blot from protein purification from sonication protein extract of SARS-CoV-2-RBD antigens. Well 1: Magic Marker XP. Well 2: pEV. Well 3: Lysate after cell shearing. Well 4: Wash 1. Well 5: Wash 2. Well 6: Concentrated eluate (PES filter). Well 7: Concentrated eluate (cellulose filter). Well 8 pSIP_3014_LysM_RBD_His cell pellet

3.2.5.3 Cell disruption using FastPrep 24 glass beads

Seeing the poor results of both 3.2.5.1 and 3.2.5.2, cell disruption using glass beads was then explored. *L. plantarum* harboring pSIP_3014_LysM_RBD_His was and prepared as described in Section 2.13.3. After cell lysis, the protein extract was purified by FPLC. The eluate from protein purification was concentrated in a Protein Concentrator tube by centrifugation with a PES filter. Western blot analysis was used to detect SARS-CoV-2 antigens in the concentrated eluate. The eluate was added to an SDS-PAGE gel before transferred to a membrane and blotted. The antigens were hybridized with specific antibodies using the SNAP i.d. immunodetection system and prepared for visualization with chemiluminescence.

Figure 3.15 shows the UV curve during the FPLC protein purification. The A peak is the peak following the addition of washing buffer and is expected. The observed B peak is sharp and larger than earlier observed in his study, followed by the gradual rise of the increasing imidazole concentration.

Figure 3.16 shows strong bonds in lysate and cell pellet (well 3 and 6). The theoretical molecular weight of the protein (pSIP3014_LysM_RBD_His) is 45 kDa. The observed bonds here are a little higher than expected, and there are no bonds in either pEV, wash or eluate.

Multiple Bradford Assays was done to the concentrated protein eluates, showing a protein concentration ranging from 40 – 70 µg/mL after repeated attempts.

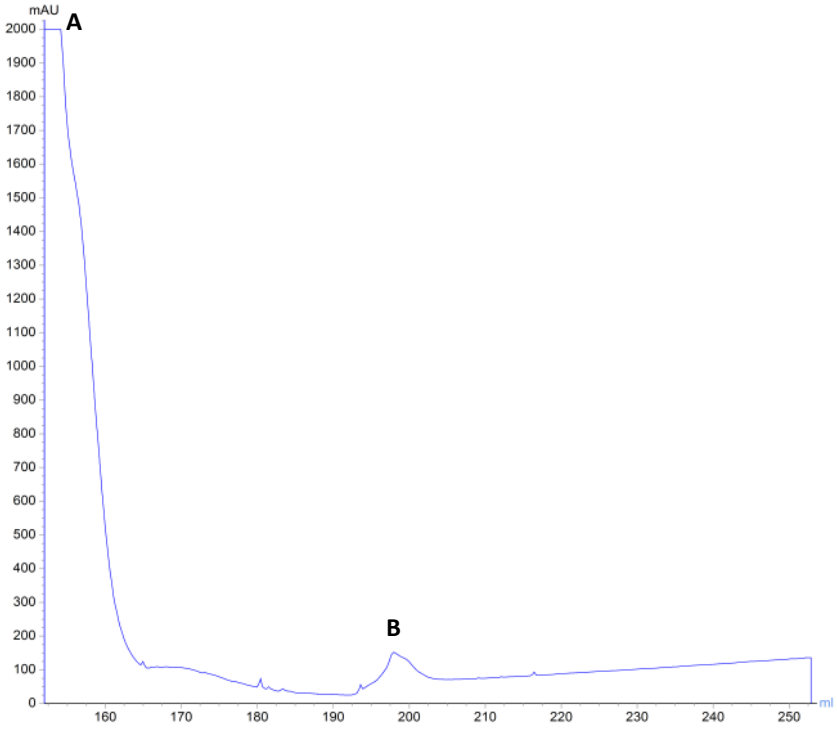


Figure 3.15. UV-curve from Fast Protein Liquid Chromatography from protein purification of protein extract. A: Washing buffer (A buffer) is added. B: Elution buffer is added.

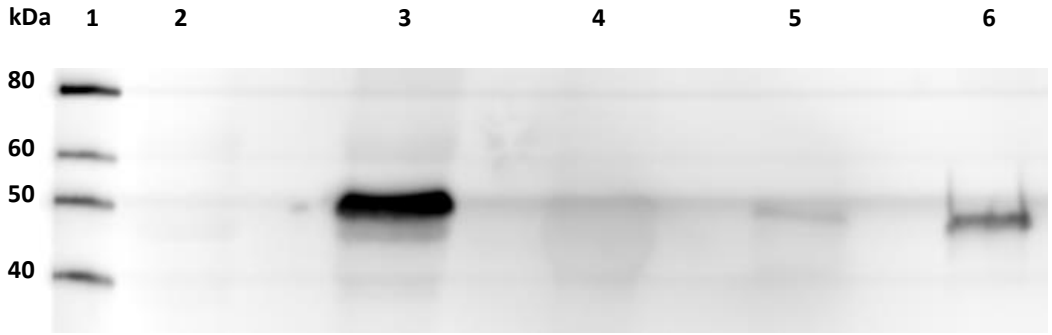


Figure 3.16. Western blot from protein purification from glass beads protein extract of SARS-CoV-2-RBD antigens. Well 1: Magic Marker XP. Well 2: pEV. Well 3: Lysate after cell shearing. Well 4: Wash. Well 5: Concentrated eluate with PES filter. Well 6 pSIP_3014_LysM_RBD_His cell pellet.

3.2.6 Flow cytometry: Detection of SARS-CoV-2-RBD antigens anchored on the surface of *L. plantarum*

L. plantarum harboring different SARS-CoV-2-RBD constructs were harvested 3 hours after induction of 25 ng/mL of SppIP and hybridized with antibodies, in the preparation of flow cytometry analysis. *L. plantarum* harboring pEV was included for negative control.

Figure 3.17 shows the flow cytometry analysis of the different constructs. The LysM anchored RBD (p3014_RBD_DC) protein showed the strongest signal, followed by the LPxTG anchored RBD (p3050_DC_RBD_cwa3001) and the lipoprotein anchored RBD (pLp_1261-RBD-DC) protein. The negative control pEV and the non-anchored LysM-fused RBD (cyt-3014_RBD_DC_His) showed the same signal strength, which was the lowest of all constructs analyzed. This indicates that spike-antigens were anchored and exposed on the cell surface.

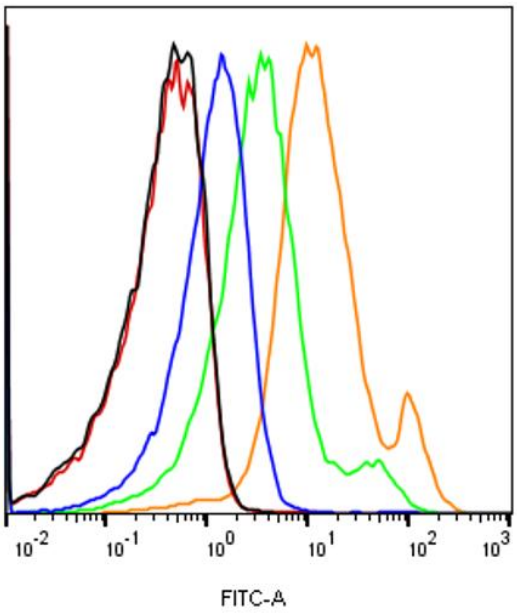


Figure 3.17. Flow cytometry analysis of *L. plantarum* with different methods of anchoring of SARS-CoV-2-RBD antigen. *L. plantarum* using different anchoring methods: Green: LPxTG anchoring (pLp_3050); Blue: lipoprotein anchoring (pLp_1261); Orange: LysM domain anchoring (p3014); Red: the not surface-bound antigen-producing bacteria (pSIP_3014_LysM_RBD_His). *L. plantarum* harboring pEV (Black) is used as negative control. Flourescence intensity is shown along the X-axis.

3.3 Anchoring of SARS-CoV-2-RBD antigens to the cell surface of living bacteria

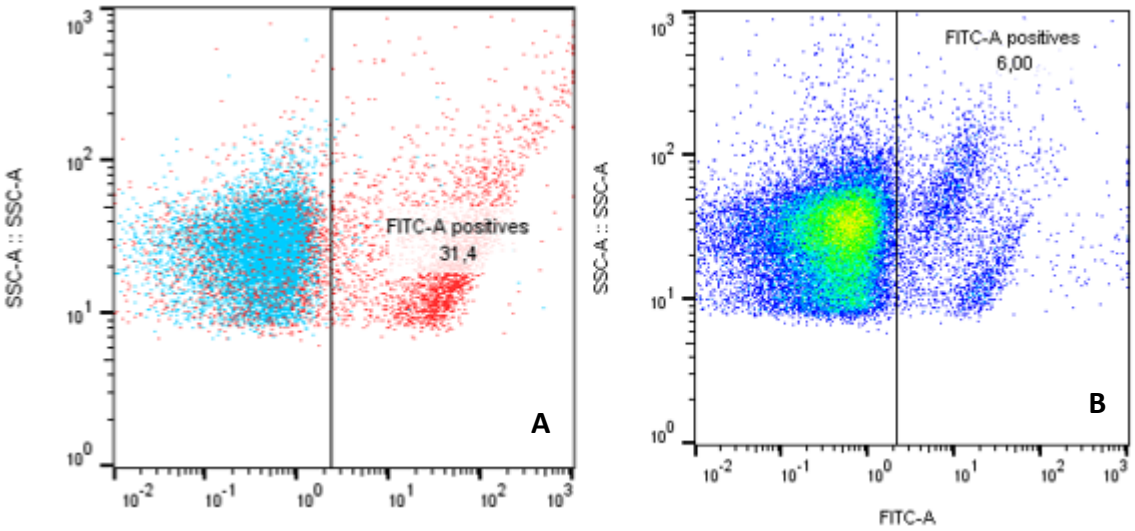
Binding studies were performed by incubating live bacteria with either purified protein solution or crude protein extract from cell disruption. Due to the low protein concentration gained from purifying protein extracts (section 3.2.5), protein extract was mainly used for incubation.

Bacteria cells used for binding studies were harvested from overnight cultures and washed in PBS, before the cell pellets were resuspended in 1 mL protein extract, with a protein concentration varying between 21,2 – 44,5 mg/mL and incubated at differing temperatures and length of time. After the incubation period was over, the bacteria cells were harvested and washed in PBS three times.

3.3.1 Binding studies with *L. plantarum*

Flow cytometry analysis was used to detect cell surface binding of LysM-fused SARS-CoV-2-RBD. Live *L. plantarum* cells were incubated 1 hour at room temperature with protein extract (total protein concentration of 44 mg/mL) and purified protein (63,86 µg/mL). The cells were then harvested, washed three times with PBS, and hybridized with antibodies.

Figure 3.18 shows the percentage of LysM-fused SARS-CoV-2-RBD antigens successfully binding to the cell surface of $3,0 \times 10^4$ bacteria cells. The binding studies were attempted multiple times with different number of bacteria cells, before the amount was decided. From the *L. plantarum* cells incubated with crude protein extract, 31,4% of cells tested positive for SARS-CoV-2-RBD, showing that the LysM-fused SARS-CoV-2-RBD antigen successfully bound to the cell surface. From the *L. plantarum* cells incubated with the purified protein solution, only 6% of all cells tested had LysM-fused protein bound to the surface. Therefore, crude protein extract was used for all later binding studies.



3.18. Flow cytometry analysis of incubation of live *L. plantarum* and crude protein extract and purified protein with LysM-fused SARS-CoV-2-RBD antigens. A) *L. plantarum* incubated with protein extract. Positive signal (red) in the right quadrant signifies detection of SARS-CoV-2-RBD antigens on the cell surface, while the negative control (light blue) is in the left quadrant. B) Live *L. plantarum* incubated with purified protein solution. Heatmap of signal strength, where the right quadrant signifies positive result, and the left quadrant no signal.

To determine if incubation temperature and incubation time influenced the successful binding of LysM-fused proteins to the cell surface of *L. plantarum*, various temperatures and times were tested. Figure 3.19 shows the results of *L. plantarum* being incubated with 1 mL of the same crude protein extract with a total protein concentration of 44,0 mg/ml. There was a small difference on the total percentage of successful binding in room temperature, with 1 (6,52%) or 2 hours (3,57%) of incubation. An incubation temperature of 4 ℃ also did not improve on the rate of successful binding. However, an incubation temperature of 37 ℃ gave a large improvement, showing that 69% of all cells analyzed gave a strong, positive signal. From this result, all future binding studies was done used 37 ℃ as the incubation temperature. Repeated binding studies using 37 ℃ as the incubation temperature did not give a as large rise in binding percentage as shown in figure 3.19, but it still showed better results than using room-temperature.

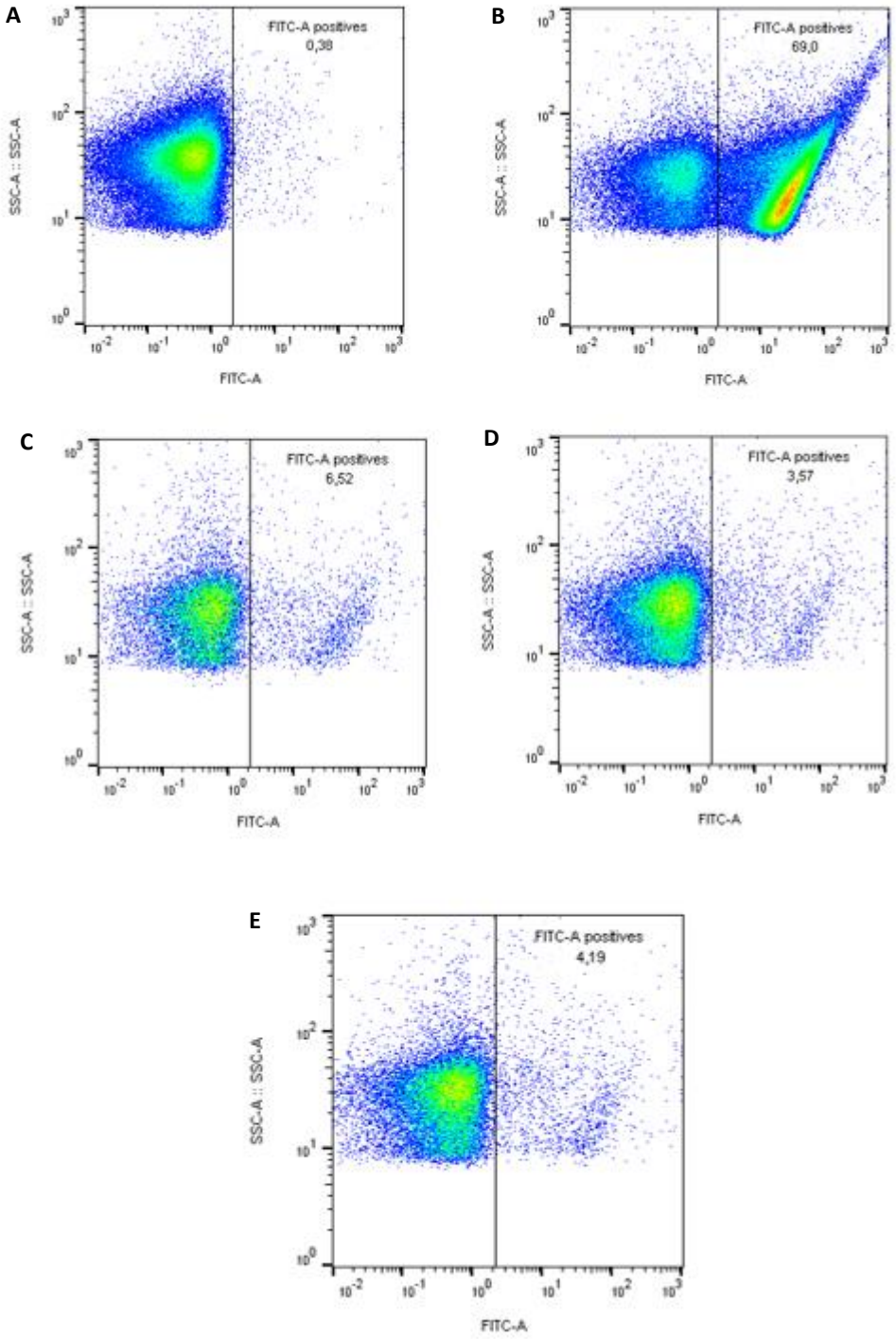


Figure 3.19. Flow cytometry analysis of *L. plantarum* surface bound LysM-fused SARS-CoV-2-RBD antigens with different incubation temperatures and times. A) Negative control incubated 1 hour at room temperature. B) Bacteria cells incubated for 1 hour at 37 ℃. C) Bacteria cells incubated for 1 hour at room temperature. D) Bacteria cells incubated for 2 hours at room temperature. E) Bacteria cells incubated for 2 hours at 4 ℃

3.3.2 LysM-fused antigens bind to a broader range of bacteria

Proteins fused with the LysM domain can in theory anchor to any cell containing peptidoglycan or chitin. For this reason, different bacterial and fungal strains (Table 3.1) was incubated with 1 mL protein extract (with a protein concentration of 44 mg/mL) and incubated for 1 hour at 37 ℃.

Table 3.1. Bacterial and fungal strains used in the binding study.

Pichia pastoris GS115	*E. coli LMG119*
Lactobacillus acidophilus	*Lactobacillus curvatus*
Lacticaseibacillus rhamnosus	*Lactiplantibacillus plantarum* WCFS1

Figure 3.20 shows that all strains tested showed surface binding of LysM-fused SARS-CoV-2-RBD antigens. The binding percentage of *P. pastoris* GS115 was at 67,4%, followed by *L. acidophilus* at 53,0% and *L. curvatus* with 50,5%. *L. rhamnosus* and *L. plantarum* WCFS1 had lower binding at 24,7% and 16,9%. *E. coli* LMG119 is a gram-negative bacterium that lacks the peptidoglycan outer layer, but still showed a small amount of binding at 3,58%. The negative control was incubated with PBS instead of protein extract and showed 0,70% binding.

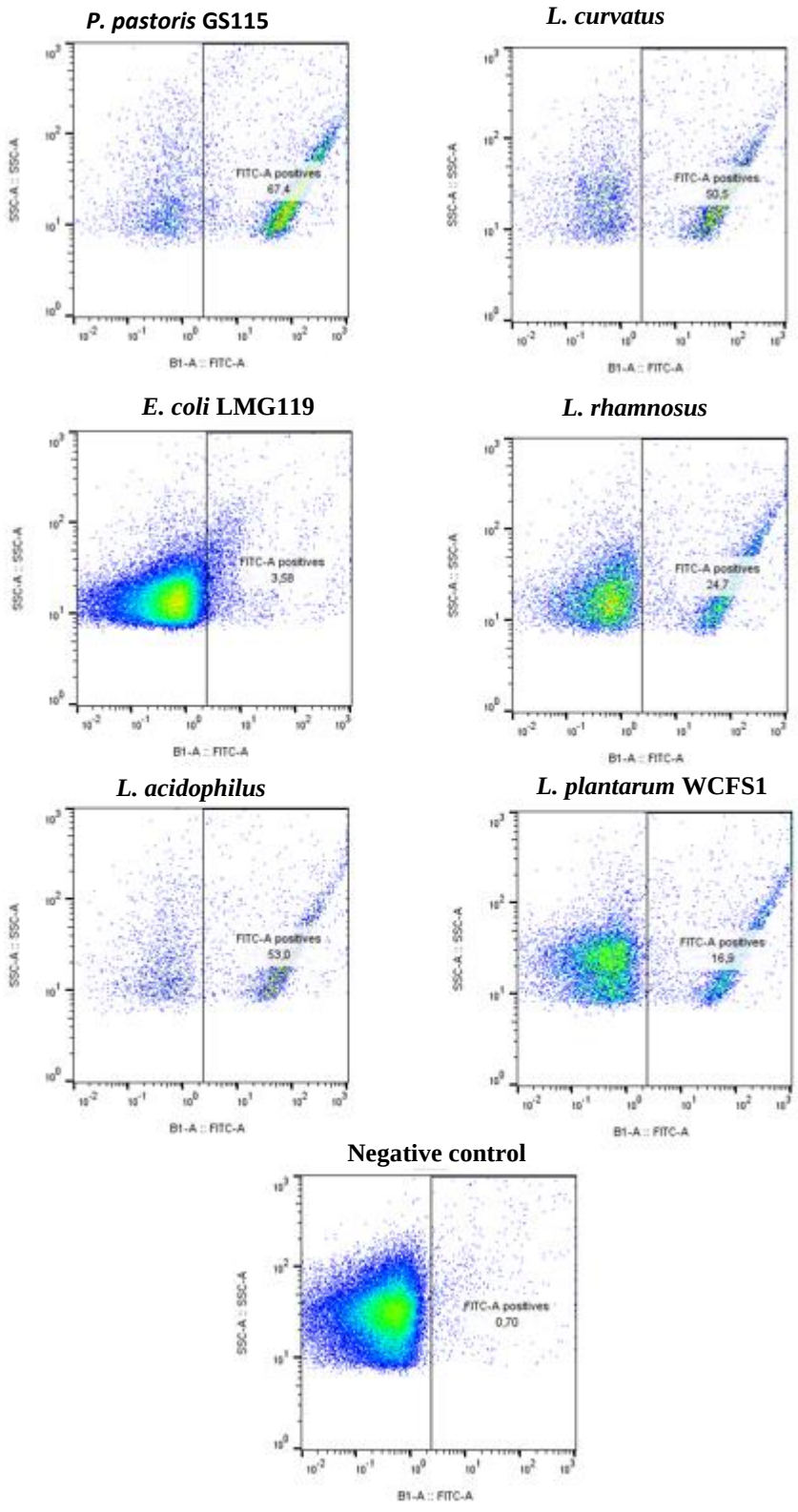


Figure 3.20. Flow cytometry analysis of surface bound LysM-fused SARS-CoV-2-RBD antigens on various bacterial and a fungal strain.

4 Discussion

4.1 Construction of plasmids for *Pichia pastoris*

In this study SARS-CoV-2 antigen for the receptor binding domain (RBD) were cloned into the pOST expression system for the production and surface display of LysM fused antigens in *Lactiplantibacillus plantarum.* The expression system of *P. pastoris* is often used in the production of heterologous proteins and *L. plantarum* have been used in multiple studies to display surface proteins and were thus used in this study (Karbalaei et al., 2020; Kuczkowska et al., 2019; Wyszynska et al., 2015). Due to the recent pandemic of the SARS-CoV-2 virus, the RBD domain was used to explore if the pOST expression system could produce SARS-CoV-2 antigens for surface display in *L. plantarum*. The hypothesis in study was that using vaccines that included the RBD domain would induce an immune response and produce antibodies that would block later infections of the SARS-CoV-2 virus.

Two different constructs were successfully created containing SARS-CoV-2 RBD domain: one containing a monomer of RBD (LysM_RBD), and the other a dimer of RBD (LysM_scRBD). Because recent studies have shown that a dimer of RBD significantly increased the number of neutralized antibodies in titers compared to the monomeric form of RBD, and increased protection in mice, if was of interest to explore this further (Dai et al., 2022; Dai et al., 2020). The antigens were anchored using the LysM anchor, as its noncovalent binding properties were utilized with the aim of creating a non-GMO vaccine and would bind to the cell wall of the fungi. Proteins containing the LysM domain are naturally found in the fungi and can bind to chitin. Chitin is a major component of the fungal cell wall, consisting of unbranched β-1, 4-linked N-acetylglucosamine, and plays an important role in the interaction between pathogenic fungi and the plant host (Kombrink et al., 2017). For these reasons, it is unknown what kind of effect producing LysM-fused proteins in *P pichia* would have.

4.1.2 Construction of plasmids for *Lactiplantibacillus plantarum*

The SARS-CoV-2 RBD antigen were successfully cloned into the pSIP expression system of *L. plantarum*. Two constructs containing a monomer of RBD were created: one containing a signal sequence for secretion out of the cell to the cell wall called pSIP3014_LysM_RBD_DC (pLp3014_LysM_RBD), and another containing a histidine tag for easier isolation and purification called pLp3014_LysM_RBD_DC_His (pSIP3014_LysM_RBD). *L. plantarum* have in previous studies been used to express antigens of *M. tuberculosis* and SARS-CoV-2 RBD antigens, and it was therefore of interest to see if the expression system could also be used to express antigens of SARS-CoV-2 RBD fused with LysM (Kuczkowska et al., 2019; Kuczkowska et al., 2017; Trondsen, 2021). The LysM anchor was used to anchor the antigens for surface display in *L. plantarum*, which binds to the cell wall of the bacterium. The two different constructs were created to maintain the aim of the study, creating a non-GMO vaccine, where the pSIP expression system was used to produce the LysM-fused SARS-CoV-2 RBD antigen. The recombinant *L. plantarum* producing the extracellular RBD antigen was created for the purpose of comparison of previous created recombinant *L. plantarum* harboring the RBD antigen using different anchoring methods (Trondsen, 2021).

4.2 Characterization of antigen production in *P. pastoris*

SDS-PAGE and western blot were used to confirm and refute the production of antigens in *P. pastoris* containing the two constructed plasmids harboring the RBD antigen (Figure 3.3, 3.4, 3.5). All surviving eletrotransformated colonies after zeocin selection (28 colonies transformed with LysM_RBD_DC and 38 with LysM_scRBD_DC) were tested. The SDS-PAGEs first returned only smudges. Optimalizations to see clear bonds lead to methanol/chloroform protein precipitation of the supernatants of harvested cultures. Seeing as there was still no strong bonds at the expected weights for both constructs (Figure 3.3), the possibility that the LysM-fused antigens stayed anchored to the fungal wall were explored. Since LysM can bind to chitin, which is a major component of the fungal cell walls of *P. pastoris*, cell disruption with glass beads were tried (Kombrink et al., 2017). The idea was that cell disruption with glass beads would crush the cell walls,

and release the LysM-fused antigens, which could be visible on SDS-PAGE gels (Figure 3.4). Proteins expressed by the expression system of *P. pastoris* may undergo glycosylation, a translational process where a carbohydrate is attached to a functional group of a molecule to form a glycoconjugate, which would result in a higher observed weight in the SDS-PAGE gels and western blots. This study was not able to determine if this was the case for the produced antigens, as there were still no clear or strong bonds observed. Contrary to using for example *E. coli* as the expression host, which results in a high expression level, the expression level of *P. pastoris* can range from low to high (Karbalaei et al., 2020). Since there had been no clear bonds up to this point, western blot of protein extract of disrupted cell was attempted multiple times to determine if the antigen were produced, but only in small amounts that were not visible from SDS-PAGE gels (Figure 3.5). The result still showed no clear bonds for the expected weights, but there were some bonds from specific strains that deserved to be further investigated. Protein purification by Ni-NTA magnetic agarose beads was performed of both crude protein extract of disrupted cells and supernatants of harvested cell cultures (Figure 3.6). As both constructed SARS-CoV-2 RBD antigens contained a His-tag, any produced antigens present would bind to the magnetic beads. The results showed no bonds and clear wells, which was repeatedly observed after multiple purification attempts.

Since every result for determining whether the transformed *P. pastoris* cells produced SARS-CoV-2-RBD antigens were negative, it was concluded that there was no production of antigens.

There are no clear answers as to why it could not be determined if there were any production of the SARS-CoV-2-RBD antigens. The expression system of *P. pastoris* is often rightfully heralded a highly successful system for synthesis of heterologous proteins (Karbalaei et al., 2020). It was determined through DNA sequencing that there were no mutations in the expressed antigen, and through antibiotic selection that the electrocompetent *P. pastoris* cells had successfully taken in the constructed plasmids. A review by Puxbaum et al. (2015) notes several challenges working with *P. pastoris* expression systems, many which can be overcome with optimalizations over time. Due to the time-consuming nature of working with *P. pastoris*, there were not enough time available for such optimalizations in this study.

4.3 Growth of recombinant *L. plantarum*

The inducer peptide SppIP were added to growing cultures of recombinant *L. plantarum* harboring different pSIP-constructs, which resulted in the production of SARS-CoV-2-RBD antigens (Figure 3.9). All induced cultures of recombinant *L. plantarum* showed clear signs of having their growth impaired compared to their uninduced counterparts, whereas the *L. plantarum* harboring pEV and the *L. plantarum* strain WCFS1 showed the same type of growth. Earlier studies have shown that growth rates may be impaired when expressing heterologous proteins, so these results where expected (Fredriksen et al., 2010; Lulko et al., 2007; Trondsen, 2021). Overexpression of the target protein may lead to stress for the bacteria, which leads to impaired growth rates. During stress the bacteria might focus more on its own survival, instead of replication, and thus leading to slower growth rates. This can be observed from the results of this study, as the *L. plantarum* strains that is induced and produces the target protein, all show impaired growth rates. The uninduced *L. plantarum* most likely does not produce the heterologous protein. It can also be seen from the impaired growth rates that the production of the target protein is most likely stressful for the bacteria. Interestingly, the growth rate of the constructed intracellular LysM-fused RBD (pSIP3014_LysM_RBD_DC_His) showed the highest growth for every other recombinant *L. plantarum* strain with different anchors tested. This indicates that it is more stressful for the bacteria to transfer the produced protein to the outside of the cell wall. The three other tested constructed utilized three different anchors, a lipoprotein anchor, a LPxTG anchor, and the LysM anchor constructed in this study. Surprisingly, the constructed LysM anchor clearly showed the worse growth rate, where the secretion of the target protein involves less steps and is not dependent on an enzyme sortase to bind to the cell wall like the LPxTG anchor is (Michon et al., 2016). This experiment was repeated twice, with the same growth curves observed.

4.4 Characterization of antigen production in *L. plantarum*

Western blot was utilized to confirm the production of antigens in *L. plantarum* harboring the two constructed plasmids (Figure 3.10). Both constructed strains showed strong bonds,

meaning they successfully produced the SARS-CoV-2-RBD antigen. There were also bonds detected from the wells containing cell pellets, however, they were not as strong as the bonds from the protein extracts from disruption with glass beads. This may suggest that even after cell disruption, a certain amount of antigens are still anchored to the cell wall. The specific western blot shown in Figure 3.10 were successful at the first try, but later attempts using either SARS-CoV-2-RBD antibodies or histidine antibodies, needed optimalizations or multiple attempts for a successful western blot. There were multiple reasons for this; monetary reasons as the price of antibodies are not low, running empty of the SARS-CoV-2-RBD antibody, which lead to the use of the histidine antibody, or a change in the BSA container used in the lab, forcing optimalizations in the % of BSA added to the blockage solution, or running empty of the iBlot materials, resulting in the use, and time-consuming optimalization, of blotting with the Tank transfer system.

4.5 Purification of antigens from *L. plantarum*

A large amount of time in this study was spent optimizing the cellular disruption protocol of the bacterial cells, and subsequent purification, of *L. plantarum* harboring pSIP3014_LysM_RBD_DC_His. As this construct was used as the expression system for the development of the non-GMO vaccine, it was of great importance that during cell lysis that largest amount possible of RBD antigen was released and made available in the protein extract, such that the amount of proteins available for purification was also as large as possible. Three methods of cellular disruption were tested. Protein extract from each method were purified, the eluate concentrated and tested by western blot.

Figures 3.11, 3.13 and 3.15 shows a UV-graph from the chromatography taken during the purification process, where the UV values corresponds to the amount of proteins that have gone through the column. Figure 3.11 shows three peaks, while the subsequent Figures 3.13 and 3.15 shows two; this is a result of an optimalization step where the first purification was done with a washing buffer B with a small amount of imidazole, to release the potential bound target protein in the column, before the elution buffer C with a high concentration of imidazole was added. As the observed elution peak C was very short and continued in a flat line, it was determined that the washing buffer B was an excess. Since it was not possible to see if the target protein was released from the column

at any point in time, subsequent purifications used only two buffers A (0 mM imidazole) and B (500 mM imidazole) added with a gradient from 0 % to 100 % over one hour, to the column. An expected elution peak would look like a tall, sharp spike, signifying that a large amount of bound protein had released from the column at once. This type of peak was not observed from any purification attempts, regardless of cellular disruption method. The flat line after the elution peak stems from the increased amount of imidazole in the added buffer, which the measured UV registers. The observed B peak in Figure 3.13 shows an improvement from the "hill" like peak in Figure 3.11, but because of the optimalization of cutting the excess washing buffer, it is hard to tell from the UV-curve alone if there was an improvement in the cellular disruption methods. Looking at the western blots from the Figure 3.12 and 3.14, there are faded bonds in the protein extracts and eluate, with strong bonds from the cell pellets. This suggests that the cellular disruption method of using either a Microfluidizer (Figure 3.12) or sonication apparatus (Figure 3.14), is not a good option for the use in this study, as most of the antigens are still bound to the cell wall. A Microfluidizer disrupts cells by pushing a single cell at a time through a tightening tube via high pressure, which "pops" the cell (Grigorov et al., 2021). This results in large cell wall fragments that the target protein could still be bound to, which could explain the large bond from the cell pellet in Figure 3.12. Sonication is a form of acoustic lysis, in which a high energy sound wave disrupts the bacteria cell, which also leaves cell wall fragments (Shehadul Islam et al., 2017). This can be seen (Figure 3.14) as the bond from the cell pellet is again the strongest, even though this figure contains a certain amount of background noise.

However, from the western blot (Figure 3.16) it can be observed that by using glass beads (or a bead mill) for cellular disruption, the strongest bond is now in the protein extract, while the bond from the cell pellet had been considerable reduced in comparison to Figure 3.12 and 3.14. The use of glass beads can lead to total disintegration of the cell membrane and cell wall, with the downside of leaving small cell debris in the solution, making the purification step tougher (Shehadul Islam et al., 2017). This aligns with the observed results from the western blot in Figure 3.16. From the UV-curve in Figure 3.15, a sharp peak B can be observed, suggesting that a certain amount of protein have been released from the column. However, since there is no washing step before the elution, this B peak

might also contain proteins that are not the target protein. This can be seen in Figure 3.16, as there is barely a bond from the concentrated eluate.

Bradford Assays were performed after each round of purification, where the highest general value (40 – 70 µg/mL) belonged to the eluate of protein extract from cellular disruption by glass beads. Interestingly, it was found that the type of filter used during protein concentration had a large impact. Eluate concentrated using a cellulose filter resulted in a total loss of proteins, where it was no longer available to be detected using western blot, so a filter of polyether sulfone (PES) was needed. This can be explained by that LysM can also bind to cellulose (Cen et al., 2017; Visweswaran et al., 2014).

4.6 Characterization of surface-display of antigens in *L. plantarum*

Flow cytometry analysis was used to investigate if the produced antigens were successfully anchored and displayed on the surface of *L. plantarum*. *L. plantarum* harboring different SARS-CoV-2-RBD constructs were analyzed (Figure 3.17). The analysis shows a stronger signal for the anchored LysM-RBD (pLp3014_LysM_RBD_DC) then the two other tested strains, which used a lipoprotein anchor (pLp1261_RBD_DC) or an LPxTG anchor (pLp3001_RBD). The intracellular LysM-RBD antigen (pSIP3014_LysM_RBD_DC_His) showed the same signal strength as the *L. plantarum* strain harboring pEV, which was expected, since the produced antigen are not supposed to be secreted for that construct. The varying signal strength amongst the different *L. plantarum* strains can most likely be explained by the different locations of the anchors on the surface of the bacteria. Antigens anchored to the cell wall would be more exposed than antigens anchored to the cell membrane, as the cell wall is on the outside of the cell membrane. The more exposed an antigen is, the more exposed it would be for the primary antibody hybridization during the analysis, and the following secondary antibody-FITC-fused hybridization, thus giving a stronger signal. Another explanation would be that the LysM anchor is localized further outside than the LPxTG or lipoprotein anchor, which could explain the differences in signal strength. However, the further out of the cell the anchored protein is, the more it is exposed for protein degradation, which could also affect the signal strength.

4.7 Binding studies of LysM-fused antigens

The strategy chosen for this study to develop a novel non-GMO mucosal vaccine was the use of LysM-fused SARS-CoV-2-RBD antigens. LysM domains in proteins could in theory bind to any cell wall containing peptidoglycan or N-acetylglucosamine, so for this reason binding studies were done (Visweswaran et al., 2014). Live *L. plantarum* cells were harvested and incubated in protein extract from cellular disrupted cells, or in concentrated eluate from the purification process. The bacteria cells were then analyzed with flow cytometry since it could detect if there were any successful binding on the bacterial surface. In the initial studies, only purified eluate was used, as that was more in-line with the vaccine development. However, the results showed barely any binding, ranging from 1 % - 8 % of cells tested positive of having FITC molecules attached to the cell surface. Eluate from all the cellular disruption methods were tested, but none showed any improvement. It was then decided to explore binding studies using only crude protein extract from glass beads, since a previous study who experienced the same problems with protein purification, found promising results using only protein extract (Målbakken, 2014). However, the initial studies again showed low percentage of FITC positive cells. A round of optimalizations were then explored, where the amount of lysozyme added before cell disruption were varied (0,1 – 1,0 mg/mL). Another optimalization were the number of bacteria cells harvested and incubated, which ranged from 1,0 x 10^7 to 5,0 x 10^5, to only 1 x 10^5. Finally, protein extract with a lysozyme concentration of 1,0 mg/mL, incubated for 1 hour at 37 °C, and the number of bacteria cells incubated for the binding study being 3,0 x 10^4, promising results could be observed (Figure 3.18). Bacteria cell incubated in protein extract resulted in that 31,4 % of the cells tested to be FITC positive, meaning that LysM-fused RBD antigens had successfully been bound to the surface of *L. plantarum*. Contrary, the bacteria cells incubated in eluate from purification of protein extract of glass beads, only gave 6 % FITC positive cells. This experiment was repeated many times, all with varying results, but incubation with protein extract always gave the highest percentage of FITC positives.

To improve the binding coverage, temperature optimalizations and incubation times were explored (Figure 3.19). Surprisingly, bacteria cells incubated at 37 °C were 69 % FITC

positive, which was nearly a 10-fold increase above the other temperatures (RT and 4℃) and times (30 minutes, 1 hour, 2 hours). As mentioned earlier in Section 1.3.1, LysM fused proteins generally binds to sites of growth and disruption on the bacterial surface. A higher temperature of 37 ℃ could provide more access to N-acetylglucosamine in the cell wall, rather than the lower temperatures of RT and 4 ℃. To further back up the temperature argument, based on a personal observation, could be that the room were the RT samples were incubated had an open window at the time, and it being early spring, lowering the RT some degrees. The only difference between the protein extract incubation sample in Figures 3.18 and 3.19 were a closed window, seemingly suggesting that the LysM-fused RBD binding were that temperature sensitive.

The claim that LysM-fused proteins could bind to any other bacteria or fungi was then explored (Figure 3.20). Different LAB, a *E. coli* strain, and a *P. pastoris* strain were then incubated with protein extract. Surprisingly, the *P. pastoris* strain showed the highest percentage of binding (67,4 %), were as *L. plantarum* WCFS1 had the lowest percentage of surface binding (16,9 %). The other LABs had a percentage of 53 % (*L. acidophilus*), 50,5 % (*L. curvatus),* and 27,4 % (*L. rhamnosus*).

4.8 Conclusion and future prospects

P. pastoris did not produce SARS-CoV-2-RBD antigens successfully in this study, whereas both constructs for *L. plantarum*, clearly did. The RBD was anchored to the surface of the bacteria using a LysM anchor. The results from SDS-PAGEs, cellular disruptions, western blots, and small-scale protein purification showed no positive results of *P. pastoris* producing the monomeric RBD or the dimeric RBD.

It would have been interesting to create new constructs of SARS-CoV-2-antigens using a different strain of *P. pastoris*, or simply removing the LysM domain from the constructed plasmids in this study, to see if the LysM domain itself was the problem. Another idea for the future would be to change promotors, from the constitutive pOST expression system used in this study, to the inducible $pAOX_1$ system that is already widely used.

The results indicate that whether the produced antigen is secreted or not, effects growth in *L. plantarum*. The *L. plantarum* strain that expressed the LysM anchored antigen had the most inhibited growth of all tested strains, whereas the *L. plantarum* strain expressing the intracellular LysM-fused antigen was the least affected. Strains harboring a lipoprotein anchored RBD and a LPxTG anchored RBD both showed stronger growth than the secreted LysM-fused RBD, but worse than the non-secreted LysM-fused RBD. The production of the non-secreted and secreted RBD antigens seems to be similar to each other, and of the different tested anchors in a flow cytometry analysis, the LysM anchored RBD antigen showed the strongest surface signal. This could be explained by the location of the different anchors in the cell wall, as the further out from the cell the anchor is, the more exposed is the antigen on the surface to its environment.

From the western blots of the different methods of cellular disruption, the use of glass beads resulted in the most released proteins from the cell wall. Cellular disruption via Microfluidizer or sonication most likely left large cell wall fragment in the solution which the LysM-fused antigen could stay bound to, while the glass beads disintegrated the cell walls. It would have interesting to try further purification with a much larger volume than used in this study, to see if it were possible to purify the antigen and gain a reasonable degree of protein from it.

Binding studies showed that the use of crude protein extract resulted in a better surface binding of the LysM-fused RBD antigen to *L. plantarum*, than the use of purified eluate. Further studies showed that the LysM-fused RBD was more than capable to bind to *P. pastoris* and other LAB, with seemingly better room for surface binding, than this study's choice of a delivery vector in *L. plantarum*. Based on the work in this study, it would seem that LysM binding to peptidoglycan or chitin is highly temperature sensitive. This would need to be further studied in the future if the work on this particular non-GMO vaccine were to be continued, since it is that noncovalent binding characteristic that is the backbone of this study's chosen strategy for developing a non-GMO vaccine. It would have been interesting to study the stability of the surface bound antigen, as vaccines may need to be transported over longer distances and be stored in specific temperatures. These are important characteristics that are necessary to know if the work on the vaccine were to continue. Another thing that would have been interesting to continue with were animal

trials, to see if the monomer or dimer of RBD would illicit an immune response and were enough to confer immunity against SARS-CoV-2.

Based on the work and results of this study, a non-GMO mucosal vaccine using *L. plantarum* as a delivery vector and expression host, is potentially viable. However, with the many problems of purifying the produced antigen in sufficient amounts from *L. plantarum*, it would be recommended to rather try methods with *P. pastoris* as the expression host.

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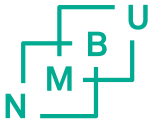

Norges miljø- og biovitenskapelige universitet
Noregs miljø- og biovitskapelege universitet
Norwegian University of Life Sciences

Postboks 5003
NO-1432 Ås
Norway