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OPTIMIZATION OF INTRANASAL COVID-19 VACCINE FORMULATION WITH *Lactococcus lactis* pNZ HCR BACTERIA AS VECTOR IN LIQUID AEROSOL PREPARATION

Optimasi Formula Vaksin Intranasal Covid-19 dengan Vektor Bakteri *Lactococcus lactis* sebagai Sediaan Aerosol Cair

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ABSTRACT
Vaccination is an effective method to suppress COVID-19 transmission, but injection-based vaccination is less effective due to its inability to induce mucosal immunity. This study aimed to determine the effects of vaccine formulations on bacteria viability and antigen expression to find the optimal formulation. Three intranasal preparation formulations (F1, F2, and F3) were created with different ingredient compositions, along with a control. Physicochemical tests were conducted on day 0 and day 14 to assess bacterial viability, and antigen expression was evaluated using the western blot method. Formula 2, containing sodium alginate (0.615%), trehalose (4.125%), polyvinyl alcohol (0.1%), and calcium chloride (5%), exhibited the best viability test results, although no significant differences were observed among the groups. The study concluded that variations in composition concentrations could affect bacterial stability, with Formula 2 showing the best results in terms of bacteria viability and antigen expression up to 14 days after formulation.

Keywords: *Formulation, intranasal vaccine,* Lactococcus lactis*, COVID-19, liquid aerosol*

ABSTRAK
Vaksinasi merupakan upaya yang efektif dalam menekan penularan COVID-19. Vaksinasi melalui rute injeksi dianggap kurang efektif karena tidak mampu membentuk imunitas mukosal yang penting dalam melawan COVID-19 dan dapat diatasi dengan vaksinasi intranasal. Penelitian ini bertujuan untuk mengetahui pengaruh formulasi vaksin terhadap viabilitas bakteri, ekspresi antigen, dan didapatkan formulasi terbaik. Penelitian dilakukan dengan membuat tiga formulasi sediaan intranasal (F1, F2, F3) dengan komposisi bahan yang berbeda, dan satu kontrol. Hasil formulasi dilakukan uji fisikokimia, uji viabilitas bakteri hari ke-0 dan 14, serta uji ekspresi antigen menggunakan metode western blot. Hasil pengujian menunjukkan tiga formulasi mampu melindungi bakteri *Lactococcus lactis* berserta antigen didalamnya selama 14 hari penyimpanan dan Formula 2 yang berisi sodium alginat 0,615%, trehalose 4,125%, polivinyl alcohol 0,1%, dan kalsium klorida 5% menunjukkan hasil uji viabilitas terbaik, walaupun hasil analisa statistik menunjukkan tidak ada perbedaan signifikan antarkelompok formulasi. Kesimpulan penelitian ini adalah variasi konsentrasi komposisi pada ketiga formulasi dapat mempengaruhi stabilitas bakteri, formula 2 menunjukkan hasil uji terbaik ditinjau dari segi viabilitas bakteri dan ekspresi antigen hingga 14 hari pasca formulasi.

Kata Kunci: Formulasi, vaksin intranasal, *Lactococcus lactis*, COVID-19, aerosol cair

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INTRODUCTION

COVID-19 is a respiratory disease that emerged in 2019 and caused a global pandemic. It is caused by the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) (Ishrath et al. 2021). The rapid mutation rate of SARS-CoV-2 has led to the emergence of numerous new variants, and the effectiveness of current vaccines against these variants remains uncertain. Therefore, it is crucial to reduce the transmission rate of SARS-CoV-2. Mass vaccination and periodic booster vaccinations are the most effective measures to provide optimal protection by increasing the number of serum antibodies with a specific immune response against SARS-CoV-2 (Baraniuk 2021; Rzymski et al. 2021).

Currently, most COVID-19 vaccines are administered via injection, either intravenously, intramuscularly, or subcutaneously. However, the injection method has certain limitations. Firstly, it is less effective in stimulating the body's mucosal immune system, which plays a crucial role as the body's first line of defense against respiratory infections, including COVID-19 (Jeyanathan et al. 2020; Russell et al. 2020; Annas and Zamri-Saad 2021; Shah et al. 2021). Secondly, vaccine administration by injection requires trained healthcare workers. Thirdly, individuals with trypanophobia, a fear of needles, may face obstacles in the vaccination process due to their excessive fear of needles (Fuad et al. 2019; Soysal et al. 2021). Therefore, future development of COVID-19 vaccines should focus on inducing mucosal immune responses (Mudgal et al. 2020; Moreno-Fierros et al. 2020). Intranasal vaccination is considered advantageous, particularly using *Lactococcus lactis* vectors, as they have been extensively studied and proven to protect inserted antigens and induce antibody formation (Azizpour et al. 2017; Mancha-Agresti et al. 2017; Shi et al. 2018). A previous study demonstrated that recombinant *L. lactis* bacteria containing the spike protein could induce systemic and mucosal antibody formation in rats (Yurina et al. 2023). The spike protein, a Highly Conserved Region (HCR), is relatively stable against mutations, making it a potential candidate for SARS-CoV-2 vaccines (Yurina 2020).

Based on these challenges, the idea of formulating an intranasal vaccine using liquid aerosol preparations emerged as an alternative. The formulation involved combining *L. lactis* NZ 3900 strain with trehalose, PVA, and calcium chloride at various concentrations. *L. lactis* is a Generally Recognized as Safe bacteria that has been widely studied as a carrier for immune vaccines (Her et al. 2015; Wyszyńska et al. 2015; Carvalho et al. 2017; Shigemori and Shimosato 2017). This study aims to identify the optimal excipient composition for the COVID-19 intranasal vaccine and assess the effects of each formulation on bacterial viability, physicochemical analysis, and antigen expression to confirm the production of SARS-CoV-2 spike protein in the formulation.

MATERIALS AND METHODS

Location and time

This research was conducted at the Pharmaceutical Laboratory, Clinical Pharmacy Laboratory, and Biomedical Laboratory of the Faculty of Medicine, Universitas Brawijaya from June to August 2022, following strict health protocols.

L. lactis pNZ HCR cultivation

L. lactis pNZ HCR strains from our previous research (Yurina et al. 2023) were cultivated on M17 agar media (Himedia, Thane West, India) supplemented with 20% lactose (Santa Cruz, Texas, USA) and incubated at 30 °C for 24 hours. *L. lactis* colonies were selected and transferred to starter cultures containing M17 Broth Media (Himedia, Thane West, India). The following day, the starter cultures were added to fresh M17 media supplemented with lactose (Sigma Aldrich, Missouri, United States), and the absorbance at 600 nm was measured. Once the optical density (OD) reached 0.8, 40 ng mL^{-1} nisin was added, and the media was further incubated at 30 °C for 24 hours. The cells were harvested by centrifugation for 10 minutes, and the pellet was washed with Phosphate Buffer Saline and resuspended in sterile water.

Vaccine formulation

In this research, the vaccine formulation used was based on the study by Nagpal et al. (2019), with modifications to the concentrations of sodium alginate, trehalose, PVA, and calcium chloride.

Table 1. Intranasal vaccine COVID-19 formulation

Substance	Control (C)	F1	F2	F3
L. lactis pNZ HCR	1×10^9 CFU	1×10^9 CFU	1×10^9 CFU	1×10^9 CFU
Sodium alginate	-	0.615%	0.615%	0.615%
Trehalose	-	2.0625%	4.125%	6.1875%
Polyvinyl alcohol	-	0.05%	0.1%	0.15%
Calcium chloride	-	2.5%	5%	7.5%
Sterile water	Ad 5 mL	Ad 5 mL	Ad 5 mL	Ad 5 mL

L. lactis pNZ HCR cells were suspended in a solution containing sodium alginate and trehalose with the appropriate concentrations shown in Table 1. The solution was then slowly dripped into 5 mL of calcium chloride and polyvinyl alcohol, according to the concentration variations in Table 1. After 12 hours of incubation, gel particles were obtained using a centrifuge. The gel particles were subsequently rinsed with sterile water three times. Finally, the gel particles were suspended in sterile water and dried using the freeze-drying method until completely dry.

Physicochemical analysis

The physicochemical analysis of the formulations included pH and viscosity measurements. pH was measured using a Schott™ pH meter, and viscosity was measured using a rotational viscometer.

Viability analysis

The prepared formulations were divided into several sample bottles (F1, F2, F3) and stored in a refrigerator at 4 °C for 14 days. Viability analysis of the recombinant *L. lactis* pNZ HCR was conducted on days 0 and 14. The samples were diluted and spread onto M17 agar media, followed by incubation at 30 °C for 24 hours. The bacterial colonies were counted the next day.

Antigen expression analysis

Antigen expression analysis was performed to determine the quantitative presence of the COVID-19 spike protein in each formulation. This analysis was conducted using the western blot method. Bacterial intracellular proteins were isolated using the sonication method, with three cycles of 5 minutes each. After isolation, the samples were subjected to SDS-PAGE electrophoresis and then transferred to a nitrocellulose membrane. The membrane was blocked overnight with a Bovine Serum Albumin solution, rinsed, and then incubated with the SARS-CoV-2 spike primary protein antigen (GenScript Biotech, New Jersey, U.S.) for 1 hour. After rinsing, the membrane was further incubated with a secondary antibody (Sigma Aldrich, Missouri, United States), and the TMB substrate was added. The resulting bands were observed and quantified using ImageJ software.

Data analysis

The obtained data were statistically analyzed using the One-Way ANOVA method in IBM SPSS® Statistics 25 software. Normality and homogeneity tests were performed using the Shapiro-Wilk and Levene tests, respectively. One-Way ANOVA was employed to determine the effect of formula variations on viability and antigen expression, with a confidence level of 95%.

RESULTS AND DISCUSSION

Physicochemical profile of the formulation

The physicochemical profiles assessed in this study were the pH value and viscosity of the resulting liquid solution. The viscosity test was conducted to determine the viscosity level of the solution. The pH test results (Table 2) indicated that the liquid solutions produced from formulations 1, 2, and 3 had the same neutral pH value of 7. The viscosity test results (Table 2) for all formulations showed values below the measurable limit of

the equipment used. This occurred because the testing instrument had a minimum viscosity limit of 3 dPa.s. As the formulation was a suspension in sterile water, the resulting preparation was expected to have a viscosity similar to water, which is around 10-3 Pa.s. Based on this test, there were no significant differences observed in the physicochemical profiles of the three formulations (Table 2).

Table 2. Results of physicochemical testing

Formulation	pH	Viscosity (Pa.s)
F1	7	10^{-3}
F2	7	10^{-3}
F3	7	10^{-3}

Viability analysis

Viability analysis was performed to determine the number of recombinant *L. lactis* pNZ HCR bacteria after the formulation process on days 0 and 14. Each analysis was repeated three times.

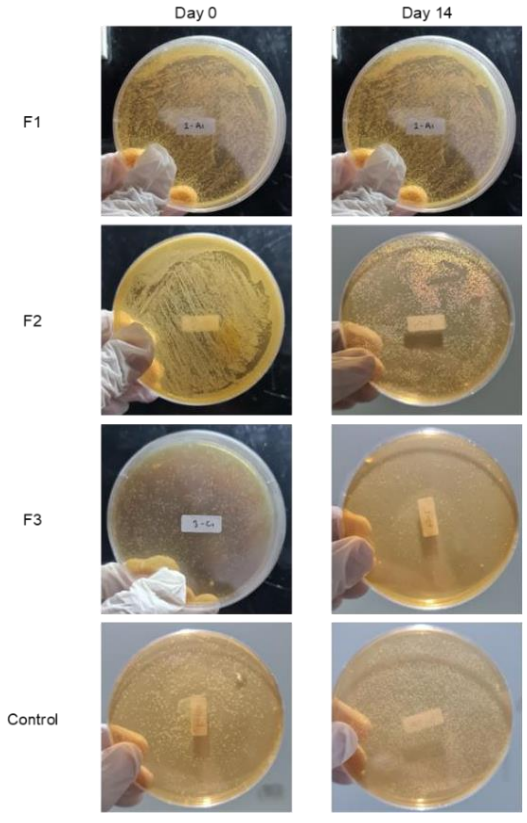

Figure 1. Bacteria culture for viability test

The viability analysis results (Figure 1) indicated that the number of recombinant *L. lactis* pNZ HCR bacteria in F1 and F2 was higher than in F3. This was evidenced by the calculation of bacterial colonies on days 0 and 14, where the number of colonies increased for F1 and F2, while the number of bacteria counted for F3 decreased. The control group also showed an increase in bacterial count from day 0 to day 14.

The viability test results graph (Figure 2) demonstrated that F1 and F2 exhibited better results compared to F3, although the One-Way ANOVA analysis showed a non-significant difference ($p > 0.05$) between F1, F2, F3, and the controls. The concentration of excipients in the formulation is one of the factors that can affect the viability test results (Nagpal et al. 2019). F1, F2, and F3 contained different concentrations of calcium chloride, trehalose, and polyvinyl alcohol, each with a distinct impact on bacterial growth. The ionic environment can interact with bacterial cell walls, particularly in gram-positive bacteria, and modulate electron flow in a substrate or enzyme, thereby effectively regulating an enzyme-catalyzed reaction (Li and Ma 2014). In our study, the variation in Ca2+ concentration appeared to influence bacterial growth, consistent with a previous study that demonstrated the inhibitory effect of Ca2+ on bacterial growth (Li and Ma 2014). Trehalose is known as a natural carbon and energy source for many organisms, including L. lactis. In this study, trehalose acted as a cryoprotectant. Our results showed that the formulation with the highest trehalose percentage yielded the lowest viability result. These findings suggest that an optimal trehalose concentration for bacterial viability is 4.125%. Moreover, the concentration of polyvinyl alcohol was also varied in our experiment. Polyvinyl alcohol serves as a coating agent. Another study demonstrated that polyvinyl alcohol could enhance the protection of bacteria during storage. After 6 hours of sequential digestion, the product exhibited improved gastric and intestinal survivability compared to unprotected controls, resulting in a 3.5-fold increase in overall survivability (Tan et al. 2019).

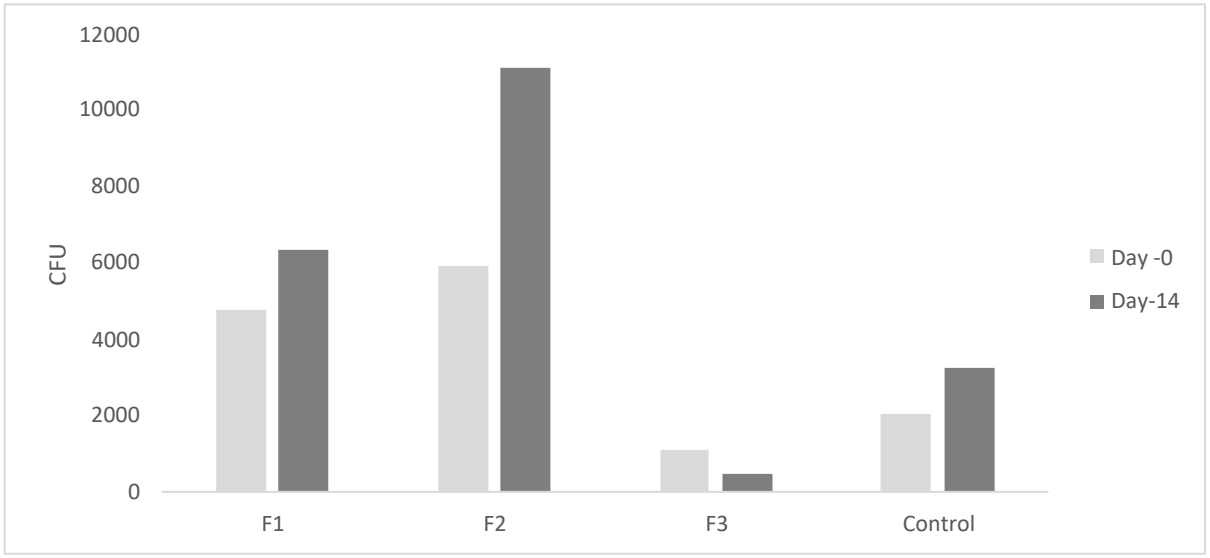

Figure 2. Viability test result. * denotes p < 0.05 F2 vs F3 (Post-Hoc Tests)

Antigen expression test

The analysis of spike protein expression, as an antigen, was conducted to confirm that the recombinant *L. lactis* pNZ HCR bacteria used in the formulation can express the spike protein of the COVID-19 virus. The quantity of antigen obtained from each formulation was assessed based on the intensity of the color band generated from the western blot test. A higher color intensity corresponded to a higher quantity of antigen produced. The test results are presented in the following graph (Figure 3).

Figure 3 shows that each formulation contained the COVID-19 spike protein, confirming that the three formulations could maintain the SARS-CoV-2 protein found in *L. lactis* bacteria. The data obtained indicated that F1 and F2 exhibited higher antigen intensity than F3. Additionally, F1 experienced an increase in antigen intensity on the 14th day, while F2 experienced a decrease in antigen intensity on the 14th day. Similar to the viability test, the concentration of excipients in the formulation could also influence the antigen expression results (Nagpal et al. 2019). This is supported by the results of the analysis using One-Way ANOVA, which showed a significant difference in antigen intensity among the three formulations (p < 0.05).

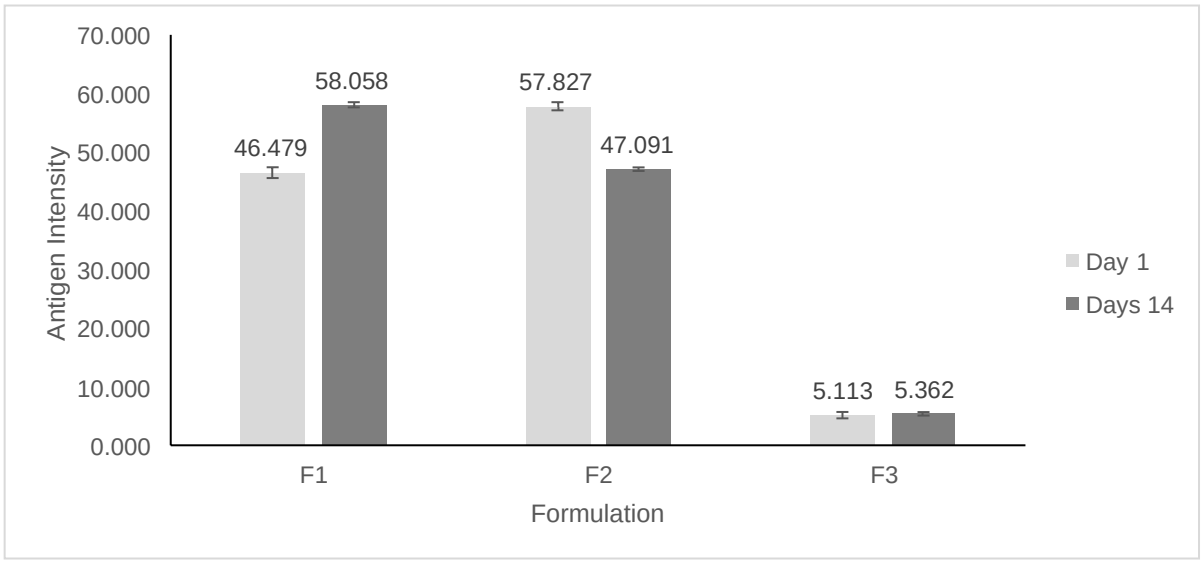

Figure 3. Antigen expression test result. * denotes p < 0.05 F3 vs F1 & F2

The best intranasal COVID-19 vaccine formulation with *Lactococcus lactis* pNZ HCR bacteria

The results of the pH test, viscosity test, viability test, and antigen expression test formed the basis for selecting the best COVID-19 intranasal vaccine formulation using recombinant *Lactococcus lactis* bacteria pNZ HCR. Among these tests, the viability test and antigen expression test were the primary considerations. The pH and viscosity tests did not reveal any differences among the three formulations; therefore, they could not be used as primary factors for selection. In the viability test, F1 and F2 demonstrated good viability compared to the control, while the viability of F3 was lower than that of the control. Consequently, F3 was eliminated as a potential candidate for the best formulation.

The viability test results also indicated that the formulation was necessary to enhance the protection of recombinant *Lactococcus lactis* pNZ HCR bacteria, particularly during the freeze-drying process (Haindl et al. 2020). This can be observed by comparing the viability of F1 and F2 to the control without any added excipients or additives. The freeze-drying method itself can cause damage to bacterial cells due to direct exposure to extreme temperatures (Mendoza et al. 2013; Rockinger et al. 2021). However, an excessive concentration of excipients or additives in the formulation is also unfavorable for bacteria, as observed in the viability test results for F3.

In general, the production of this vaccine formulation consists of one active ingredient and five excipients or additives. *Lactococcus lactis* pNZ HCR bacteria serve as the active ingredient, while the excipients used include sodium alginate, trehalose, calcium chloride, polyvinyl alcohol (PVA), and Water for Injection (WFI). Sodium alginate acts as a freeze-drying agent that ensures the stability of the active ingredients. Trehalose functions as a freeze-drying agent and stabilizing agent during the freeze-drying process. Calcium chloride acts as an antimicrobial and water-absorbing agent. PVA serves as a coating agent and stabilizing agent. WFI is used as a solvent (Rowe et al. 2009).

Based on the post hoc test results, no significant difference was found between F1 and F2 ($p > 0.05$). This indicates that both F1 and F2 can be considered equally superior to F3 in terms of antigen expression test results. Based on the results of the post hoc test, F2 was determined as the COVID-19 intranasal vaccine formulation using the best pNZ HCR recombinant *Lactococcus lactis* bacteria vector. This conclusion is also supported by the viability data, which showed that the viability of F2 was higher than that of F1.

CONCLUSION

In conclusion, the concentration of excipients in the formulations (trehalose, calcium chloride, and polyvinyl alcohol) was found to affect the viability test results and antigen expression produced from the three formulations. Excessive antigen concentration can lead to a decrease in the viability of *Lactococcus lactis* bacteria, resulting in lower antigen intensity. The optimal formulation of the COVID-19 intranasal vaccine with recombinant *Lactococcus lactis* bacteria carrier pNZ HCR was F2, which contained 0.615% sodium alginate, 4.125% trehalose, 0.1% polyvinyl alcohol, and 5% calcium chloride.

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