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Protective effects of immunization with a novel 4 recombinant pore-forming toxoid combination vaccine in a rabbit model of systemic methicillin-resistant *Staphylococcus aureus* infection

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Purpose: *Staphylococcus aureus* is a Gram-positive bacterium that most frequently acquires antibiotic resistance. As an opportunistic pathogen, it can cause conditions such as bacteremia, sepsis, and myocarditis. Due to the social need for a vaccine against methicillin-resistant *Staphylococcus aureus* (MRSA), many research groups have been designing and studying vaccines for decades. In this study, we developed a multivalent vaccine and evaluated its efficacy by applying a novel adjuvant, β-glucan.

Materials and Methods: A vaccine composed of four pore-forming toxins from *S. aureus* was administered to rabbits 3 times, after which they were challenged with *S. aureus* USA 300 LAC strain. We measured changes in the rabbits' body weight to monitor systemic adverse reactions and analyzed the total immunoglobulin G antibody titer against the four antigens using enzyme-linked immunosorbent assay. For each rabbit, the number of abscesses and colony-forming units (CFU) in the kidneys were measured.

Results: In all vaccinated groups, strong antibody responses against the four antigens were observed. After challenging with MRSA, the vaccinated groups showed less weight change compared to the non-vaccinated groups (average 5.7% versus 13.5%). Additionally, the number of renal abscesses was significantly lower in the vaccinated groups, with three individuals in group 1 (four antigens adjuvanted with β-glucan_PK1) showing no abscess formation. The number of bacteria identified in the kidneys was also statistically significantly lower in the vaccinated group compared to the non-vaccinated group.

Conclusion: We demonstrated that the four toxoid antigens we selected can protect against *S. aureus* infection in a rabbit model and that β-glucan could be used as an immune enhancer. Overall, our study shows that new antigen combinations can induce protective immunity in animal models and that a toxin-based vaccine can help control bacterial colonization.

Keywords: Toxin, Vaccine, Rabbit model, Methicillin-resistant *Staphylococcus aureus*

Introduction

Staphylococcus aureus is a gram-positive pathogen that causes a broad spectrum of diseases in humans, ranging from skin and soft tissue infections to severe, life-threat-

ening conditions such as sepsis, pneumonia, endocarditis, and toxic shock syndrome [1,2]. Additionally, this organism has spread and now causes both community-acquired infections and serious nosocomial infections. In the past, staphylococcal infections were effectively treated with anti-staphylococcal antibiotics. However, *S. aureus* has developed increasing resistance to numerous antibiotics, including methicillin and vancomycin [3-6]. Furthermore, despite selective antibiotic treatments, the prognosis remains poor due to the limited efficacy of these antibiotics against many staphylococcal toxins. The pathogenic versatility of *S. aureus* is largely attributed to its ability to produce a wide range of virulence factors, with exotoxins playing a pivotal role in evading the host immune system by targeting and destroying host immune cells. For example, pore-forming toxins, such as bicomponent leukocidins and hemolysin alpha (Hla), can destroy phagocytic immune cells, platelets, and red blood cells [7,8]. The global emergence and rapid spread of methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Staphylococcus aureus* underscore the urgent need for the discovery and development of novel therapeutics, including an effective vaccine, to counteract these problems. Therefore, vaccine development is a critical and viable tool for controlling and overcoming these challenges.

Over the past 2 to 3 decades, numerous studies and clinical trials have been conducted in an effort to develop a staphylococcal vaccine, yet no effective vaccine is currently available. Although initial vaccine development efforts focused on capsules and surface proteins, it has become clear that targeting cell wall proteins alone is insufficient to induce effective immune responses against *S. aureus*. An antitoxin approach must be integrated into the vaccine development strategy [9-11]. A major challenge in creating a successful vaccine against *S. aureus* is the incomplete understanding of protective immune mechanisms and the lack of reliable biomarkers that indicate durable and effective protective immunity against *S. aureus* infections in humans [2,12]. This challenge is further compounded by limited information regarding the specific host immune responses necessary to protect against invasive *S. aureus* infections [2].

In our previous study, we identified the combination of HlgA (gamma-hemolysin component A), LukS (leukocidin S), Hla_{H35L} (Hla, mutant at position H35L), and $LukA_{E323A}B$ (leukocidin A, mutant at position E323AB) antigens as a potentially optimal vaccine formulation for protecting human red blood cells (RBCs) and polymorphonuclear neutrophils (PMNs) from staphylococcal pore-forming toxins [13]. We hypothesized that an effective *S. aureus* vaccine would require not only these toxin antigens but also additional components capable of eliciting immune responses that prevent staphylococcal colonization. To further investigate this, we revisited the protective efficacy of a quadrivalent vaccine against MRSA systemic infection, incorporating adjustments to the toxoid dose and immunization schedule. Additionally, we examined the matched adjuvant effects of β-glucan, recognized for its ability to activate innate immunity and promote T cell activation.

Materials and Methods

Animals and immunization

Twenty-five outbred New Zealand White rabbits (1.7–2.2 kg; SamDako, Gwangju, Korea) were enrolled in this study. The rabbits were divided into five groups based on the mixed adjuvant used in the study vaccine as follows (Table 1): group 1 received the vaccine with β-glucan PK1 (purified β-glucan from fermented oats [provided by With-BioCospharm, Namyangju, Korea]), group 2 received β-glucan PK1 combined with alum (alhydrogel), group 3 received purified β-glucan from raw oats only, group 4 received alum (alhydrogel) only, and group 5 served as the control group, receiving phosphate-buffered saline (PBS). The rabbits were immunized at the dorsal lumbar region 3 times on days 0, 14, and 42 with vaccines containing recombinant pore-forming toxoids (LukS; 30 μg, $LukA_{E323A}B$; 40 μg, Hla_{H35L}; 25 μg, HlgA; 40 μg). The toxoid vaccine was prepared using the same methods as in our previous study [13].

Safety evaluation

After vaccination, all rabbits were closely monitored for general appearance, activity, feeding condition, injection site morphology, and weight loss. Daily weight changes were recorded for 7 days following each vaccination. Additionally,

Table 1. Classified group by each adjuvant formulation

Group	Adjuvant	Adjuvant dose	No. of subjects
1	β-glucan_PK1	1,350 μL/rabbit	5
2	β-glucan_PK1 + alhydrogel	1,350 μL/rabbit of each adjuvant dose	5
3	β-glucan (normal)	1,350 μL/rabbit	5
4	Alhydrogel	1,350 μL/rabbit	5
5 (Control)	Phosphate-buffered saline	1,350 μL/rabbit	5

the survival of both vaccinated and control rabbits was observed throughout the study period.

Humoral immunity assessments

Blood was collected from the rabbits on days 7, 14, 21, 28, and 37 after vaccination, and on day 49 after the challenge. Final antibody levels against the toxins were measured using the enzyme-linked immunosorbent assay (ELISA) method as follows: ELISA plates (Falcon, Franklin Lakes, NJ, USA) were coated with 100 μL of each antigen stock solution (5 μg of each antigen dissolved in 5 mL of coating buffer [50 mM sodium bicarbonate, pH 9.4]) and incubated overnight at 4°C. After removing the supernatant, each well was washed 3 times with 200 μL of washing buffer (PBS with 0.05% Tween 20). Each well then received 100 μL of blocking buffer (PBS with 0.2% bovine serum albumin [BSA]) and was incubated at room temperature for 1 hour, followed by three washes with 200 μL of washing buffer. Threefold serially diluted samples (starting from 1:180 for rabbit sera) were applied to the plates and incubated at room temperature for 2 hours. Horseradish peroxidase-conjugated anti-rabbit immunoglobulin G (IgG) antibody (50 μL per well; Santa Cruz Biotechnology, Santa Cruz, CA, USA) was added to each well and incubated at room temperature for 2 hours. For color development, 50 μL of tetramethylbenzidine solution (TMB; Thermo Fisher Scientific, Waltham, MA, USA) was added to each well and incubated for 1 to 3 minutes. After the addition of the stopping solution (50 μL, 0.5 N HCl), the optical density (OD) was measured at 450 nm using an ELISA reader (MULTISKAN GO; Thermo Fisher Scientific). Endpoint titers of each antigen-specific antibody were expressed as the reciprocal $\log_2$ of the last dilution yielding a positive color change (OD 450 nm of ≥0.3).

Challenge with MRSA strain

The *S. aureus* strain USA 300 LAC, previously characterized [14], was grown to mid-logarithmic phase (OD at 600 nm [OD600] of 0.8 to 1.5) in tryptic soy broth at 37°C with shaking at 180 revolutions per minute, as described previously [9].

In a high-challenge dose experiment, rabbits (five in the control group and 20 in the vaccinated group) were challenged. On day 49, the rabbits were injected via the ear vein with *S. aureus* USA300 LAC (7×10^7 colony-forming units (CFU)/kg *S. aureus* in 100 μL of PBS with 0.01% BSA) and monitored every 12 hours, including body weight changes, for 10 days. All immunized rabbits were euthanized on day 10 post-infection. Kidneys were aseptically isolated and examined for morphology, weight, and the presence of abscesses. Portions of the isolated renal tissue (cut into pieces less than 0.5 cm) were homogenized in 0.9% saline, and CFU titers were measured by plating serial dilutions on blood agar.

Ethics statement

This animal experiment was conducted in accordance with the institutional guidelines and approved by the Pusan National University Institutional Animal Care and Use Committee (PNU-IACUC; approval number: PNU-2021-2919). Every effort was made to minimize the suffering of the animals.

Statistical analysis

The results are presented as the mean±standard deviation. Statistical significance was determined using an unpaired Student t-test or a log-rank test with GraphPad Prism ver. 8.4.0 (GraphPad Software, San Diego, CA, USA). Differences were considered significant when the p-value was equal to or less than 0.05.

Results

Safety after immunization

After vaccination, all rabbits exhibited stable activity levels and good feeding conditions across all groups. No weight loss was observed within 7 days post-immunization in any of the groups. Additionally, none of the rabbits showed behavioral changes after immunization, and no deaths occurred prior to the septic challenge. There were also no differences in response based on the order of vaccination.

Antibody responses

All humoral responses showed an increasing trend with successive vaccinations in the study groups. The final average reciprocal IgG titer ($\log_2$) for LukS was 10.24 in group 1, 10.01 in group 2, 8.43 in group 3, and 9.71 in group 4. The final average reciprocal IgG titer ($\log_2$) for $LukA_{E323A}B$ was 9.14 in group 1, 11.19 in group 2, 8.10 in group 3, and 9.87 in group 4. The final average reciprocal IgG titer ($\log_2$) for Hla_{H35L} was 12.56 in group 1, 11.82 in groups 2 and 3, and 11.93 in group 4. The final average reciprocal IgG titer ($\log_2$) for HlgA was 9.71 in group 1, 10.45 in group 2, 9.07 in group 3, and 9.92 in group 4. Based on these results, we found that the levels of LukS and Hla_{H35L} IgG were highest in group 1, while $LukA_{E323A}B$ IgG and HlgA IgG were highest in group 2 (Fig. 1).

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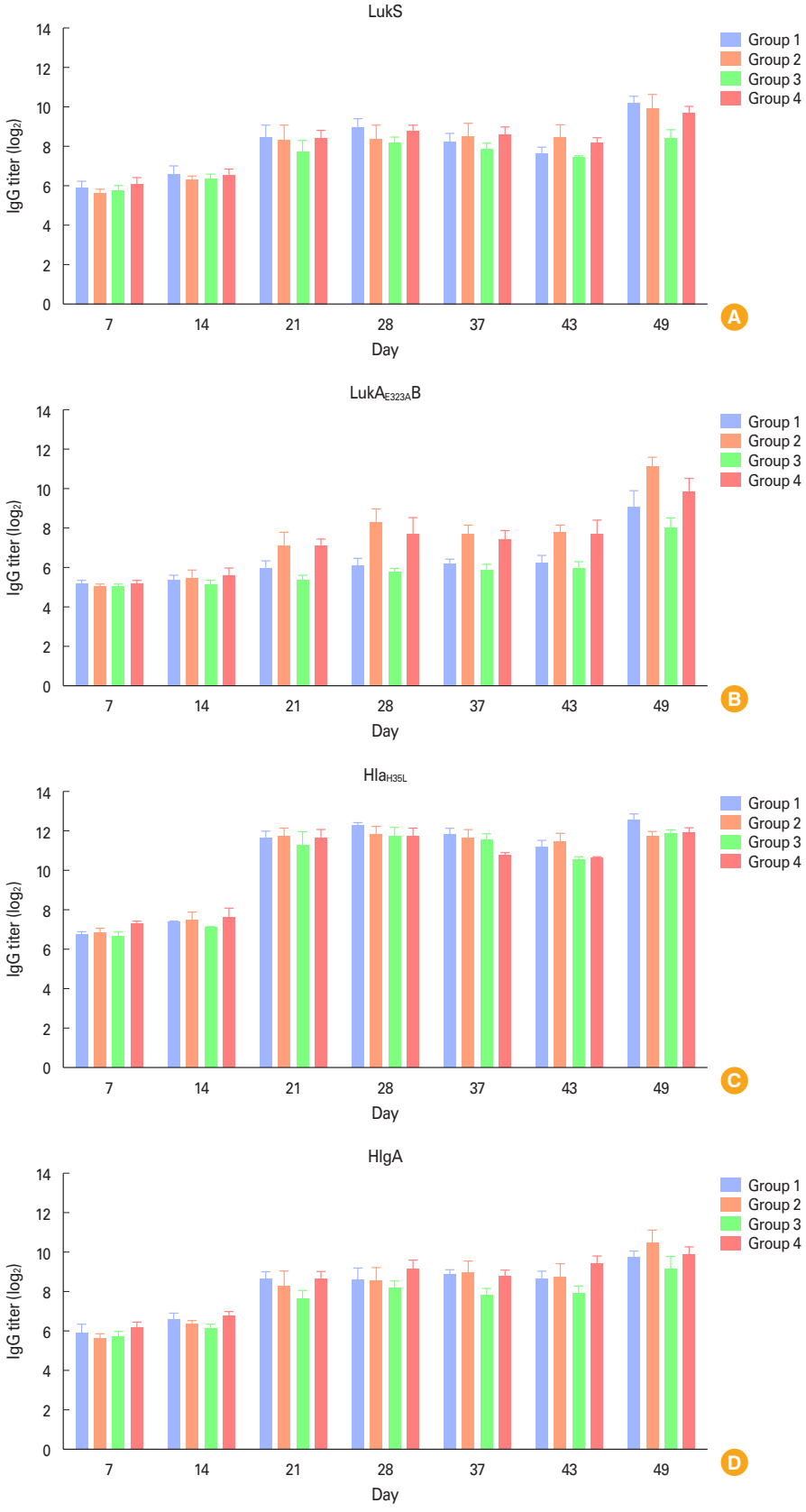

Fig. 1. (A–D) Humoral antibodies against to toxoid antigens by vaccination in each group: LukS, $LukA_{E323A}B$, Hla_{H35L}, and HlgA. Group 1: 4 antigens+β-glucan (PK1_purified from fermented oats). Group 2: 4 antigens+β-glucan (PK1)+aluminium hydroxide. Group 3: 4 antigens+β-glucan (purified from raw oats). Group 4: 4 antigens+aluminium hydroxide. IgG, immunoglobulin G.

Results after septic challenges

Weight changes and survival rates after septic challenge

On average, group 1 showed a 3.37% weight loss (2,851 g before challenge → 2,755 g after challenge on day 7), group 2 showed a 4.47% weight loss (2,997 g → 2,863 g on day 7), group 3 showed a 6.77% weight loss (3,085 g → 2,876 g on day 7), group 4 showed a 8.17% weight loss (3,074 g → 2,823 g on day 7), and group 5 showed an 13.51% weight loss (3,175 g → 2,746 g on day 7) (Fig. 2). When comparing weight loss patterns between groups, group 1 showed a slight decrease with mild individual differences, while the control group (group 5) displayed a clear downward trend after the septic challenge (Fig. 2). All rabbits in groups 1 and 4 survived the study period. However, there were deaths in one rabbit in group 2 (20% mortality), two rabbits in group 3 (40% mortality), and three rabbits in group 5 (60% mortality) between 7 days and 10 days post-challenge.

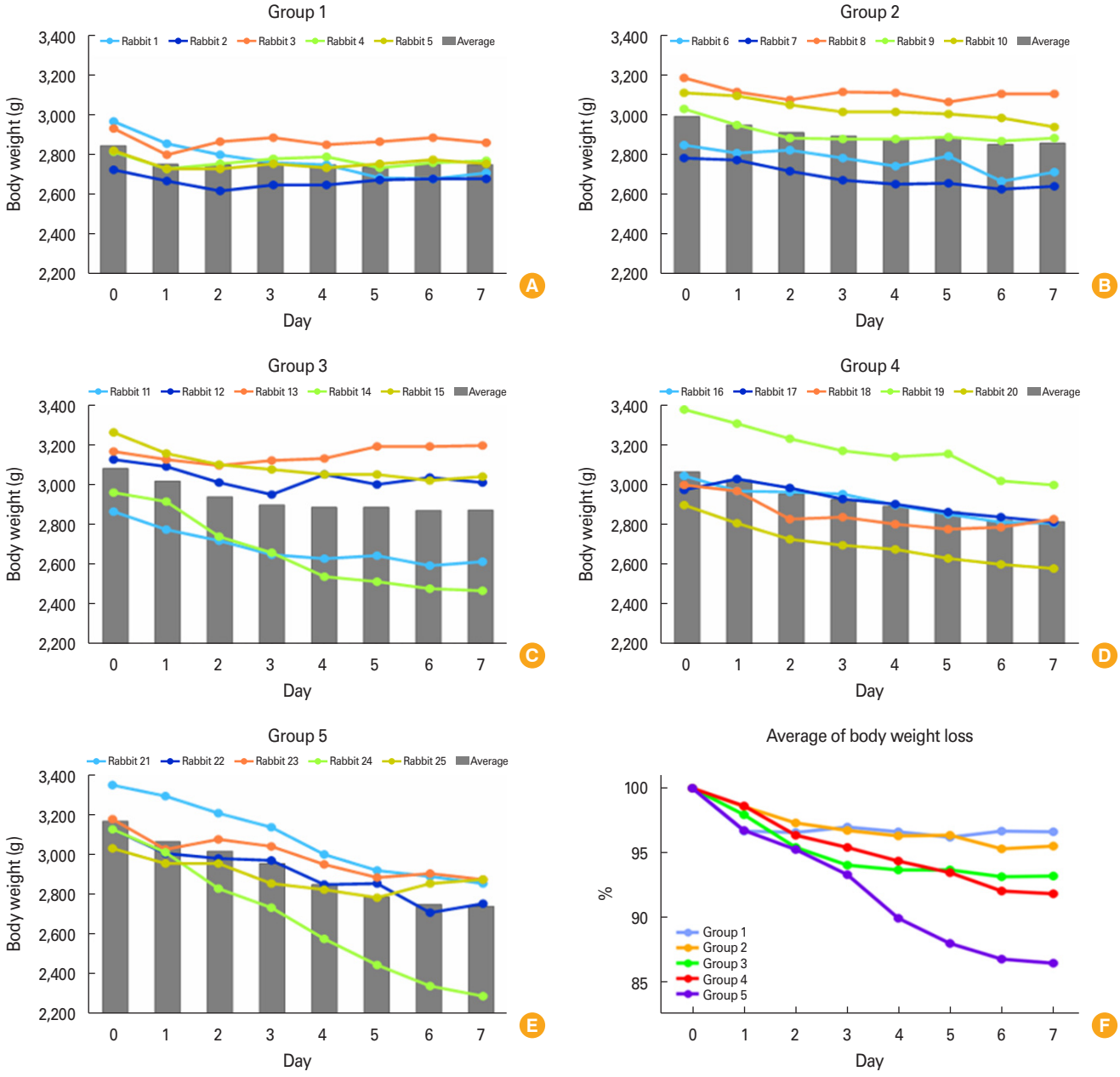

Fig. 2. Weight changes after septic challenges in each group. **(A)** Group 1: 4 antigens+β-glucan (PK1_purified from fermented oats). **(B)** Group 2: 4 antigens+β-glucan (PK1)+aluminium hydroxide. **(C)** Group 3: 4 antigens+β-glucan (purified from raw oats). **(D)** Group 4: 4 antigens+aluminium hydroxide. **(E)** Group 5: control. **(F)** Average of body weight loss; comparison of average weight loss for each group from day 0 to day 7.

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Renal abscess formation

Gross examination confirmed renal abscess formation in all groups as follows: two renal abscesses were found in group 1, four in group 2, all cases in group 3, three in group 4, and all cases in group 5. Notably, no abscesses were found in cases 1, 3, and 4 in group 1. In contrast, marked renal abscess formations were observed in all cases in groups 3 and 5 (Fig. 3).

Total CFU

The vaccine's effect on bacterial dissemination and colonization in the kidneys was observed. On day 10 post-infection, all surviving rabbits were euthanized, and their kidneys were harvested and homogenized. In cases where rabbits died, the kidneys were removed immediately after death, harvested, and homogenized. CFUs in the kidneys were measured by serial dilution and plating on tryptic soy agar. The statistical significance of CFU differences was assessed using an unpaired Student t-test. When comparing the total average CFU/100 mg (homogenized renal tissue) between groups, we found that total CFU correlated with gross findings of renal abscess

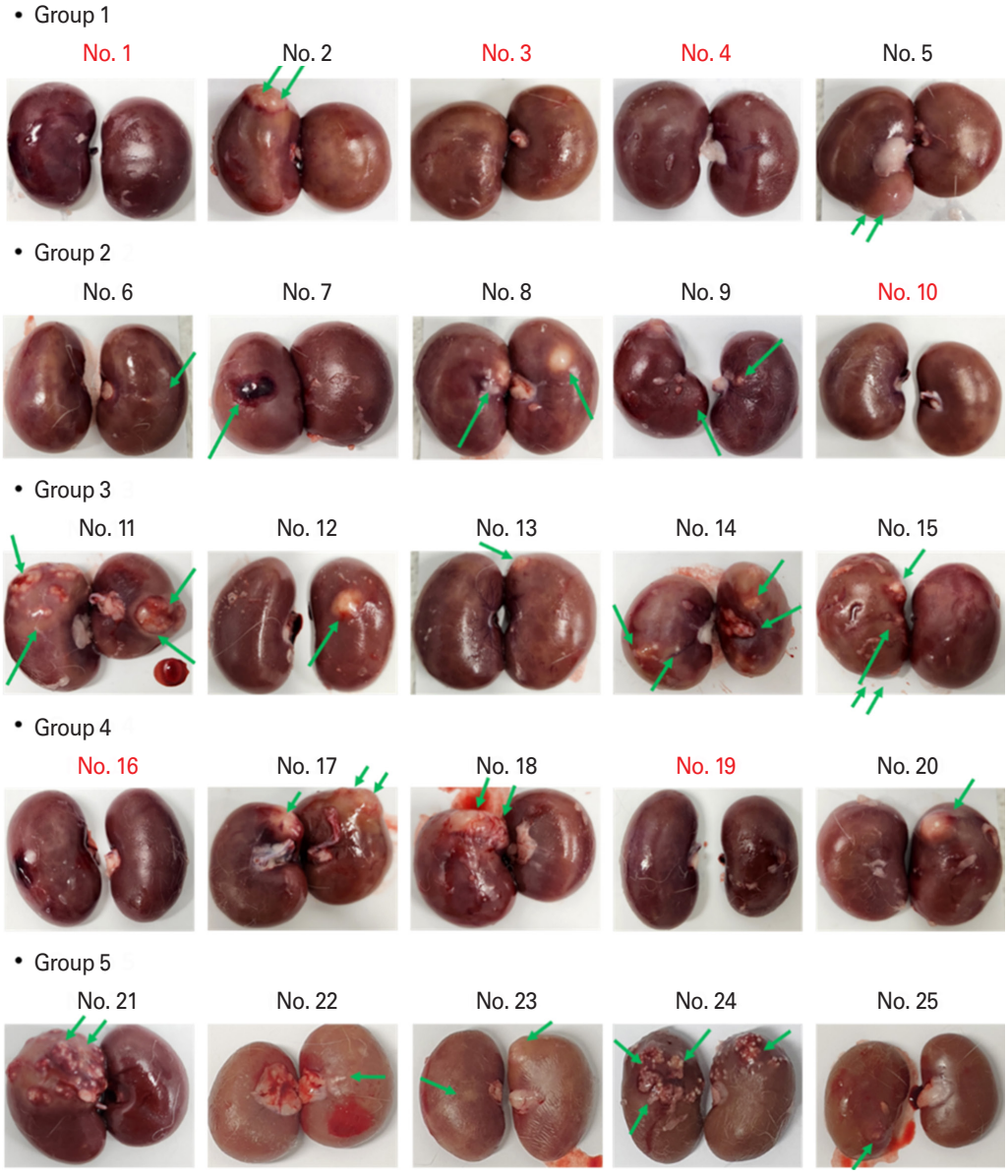

Fig. 3. Renal abscess formations after septic challenges in each group; Staphylococcal renal abscesses are indicated with green arrows, and no abscesses are indicated with red number. Group 1: 4 antigens+β-glucan (PK1_purified from fermented oats). Group 2: 4 antigens+β-glucan (PK1)+aluminium hydroxide. Group 3: 4 antigens+β-glucan (purified from raw oats). Group 4: 4 antigens+aluminium hydroxide. Group 5: control.

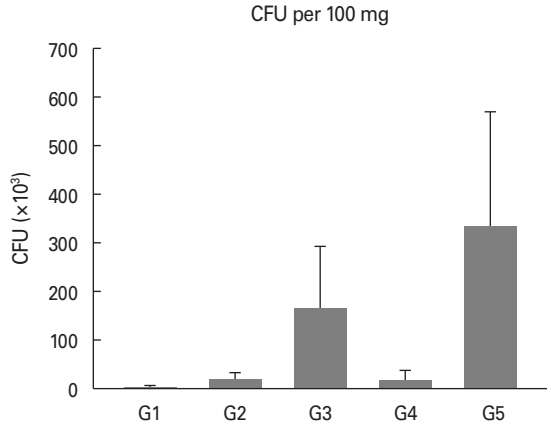

Fig. 4. Total average colony-forming units (CFU)/100 mg of isolated kidney after septic challenges in each group. Group 1: 4 antigens+β-glucan (PK1_purified from fermented oats). Group 2: 4 antigens+β-glucan (PK1)+aluminium hydroxide. Group 3: 4 antigens+β-glucan (purified from raw oats). Group 4: 4 antigens+aluminium hydroxide. Group 5: control.

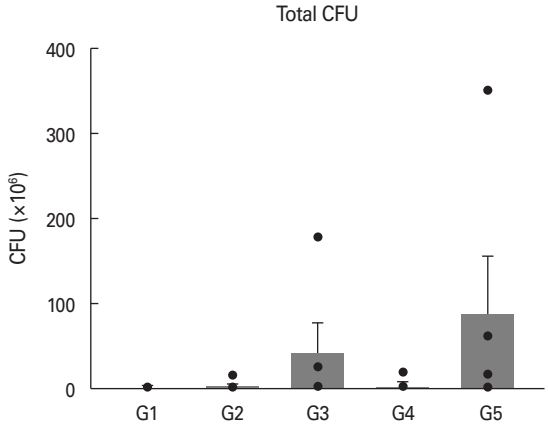

Fig. 5. Total colony-forming units (CFU) of isolated individual kidney after septic challenges in each group. Group 1: 4 antigens+β-glucan (PK1_purified from fermented oats). Group 2: 4 antigens+β-glucan (PK1)+aluminium hydroxide. Group 3: 4 antigens+β-glucan (purified from raw oats). Group 4: 4 antigens+aluminium hydroxide. Group 5: control.

formation. Additionally, the bacterial burden was significantly lower in vaccinated rabbits than in control rabbits (Fig. 4). In fact, no detectable CFUs were found in group 1 (three cases; cases numbers 1, 3, 4), group 2 (one case; case number 10), and group 4 (two cases; cases numbers 16, 19) where there was no gross evidence of renal abscess formation (Fig. 5).

Discussion

In our previous study, we identified a combination of HlgA, LukS, Hla_{H35L}, and $LukA_{E323A}B$ antigens as a promising vaccine formulation for protecting against staphylococcal pore-forming toxins. In this study, we evaluated the quadrivalent vaccine against MRSA, adjusting the toxoid dose and immunization schedule, and explored the adjuvant effects of β-glucan. Our results demonstrated that the quadrivalent vaccine, particularly when combined with β-glucan, provided strong protection, as evidenced by the survival of all vaccinated rabbits and a significant reduction in renal abscess formation. Notably, β-glucan purified from fermented oats showed superior effectiveness in inhibiting intracellular MRSA colonization compared to alum. These findings suggest that β-glucan could be a valuable adjuvant in MRSA vaccine development, although further research is needed to optimize antigen dosing and understand the underlying mechanisms of β-glucan action.

S. aureus, traditionally considered an extracellular pathogen, has recently been recognized to also act as an intracellular pathogen [15]. Indeed, antibody-mediated opsonophagocytosis is ineffective at killing intracellular *S. aureus*. This indicates that bacterial clearance is not fully achieved by humoral and opsonophagocytic antibodies alone and suggests that both humoral and cell-mediated immunity (CMI) are necessary to induce effective protection against intracellular *S. aureus* infections [16,17]. Identifying suitable vaccine adjuvant molecules that can activate host innate immunity when co-injected with already identified *S. aureus* antigens could potentially induce T-cell-mediated cellular immunity. To achieve this goal, we must understand which *S. aureus* vaccine antigens have been screened thus far, identify appropriate animal models for *S. aureus* vaccine development, and consider the biochemical characteristics of promising vaccine adjuvants based on recent published data. Additionally, increased knowledge about the mode of action of already approved vaccines, as well as newly developed and promising adjuvants, is essential for the development of an effective *S. aureus* vaccine.

According to previous studies and vaccine development experiences, many researchers suggest that an effective anti-staphylococcal vaccine should protect hosts from staphylococcal toxins while simultaneously inhibiting staphylococcal colonization [18]. Several recent studies have reported that anti-toxin antibodies may enhance protective efficacy against severe staphylococcal infections. For example, the Hla toxin, which increases lytic activity in PMNs, RBCs, and platelets by binding to the specific cell receptor ADAM10 and forming

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transmembrane pores, is a major toxin associated with the severity and mortality of *S. aureus* infections. Therefore, anti-alpha-toxin monoclonal antibodies could reduce the severity and mortality of MRSA infections. Several *in vivo* studies have shown that alpha-hemolysin-neutralizing antibodies provide protective effects in severe infections, including pneumonia, soft-tissue infections, and bacteremia [19,20]. Additionally, bi-component pore-forming toxins such as LukS and LukF form functional pores in immune cells, including PMNs. *S. aureus* can be neutralized by antisera against vaccine candidates based on the LukS and LukF subunits of Panton-Valentine leukocidin, but not by anti-Hla neutralizing antibodies. Thus, a combination of Hla_{H35L} with LukS or LukF as vaccine components could be targeted to MRSA infections. Furthermore, alpha-toxin (Hla) and leukocidin AB (LukAB), critical molecules secreted by *S. aureus* biofilms that inhibit macrophage phagocytosis and promote cytotoxicity, are potential targets. Research by Scherr et al. [21] reported that loss of LukAB and alpha-toxin expression resulted in enhanced *S. aureus* biofilm clearance in a mouse model of orthopedic implant infection. This suggests that LukAB and alpha-toxin could be therapeutically targeted to facilitate biofilm clearance in humans. Moreover, HlgA is a virulence factor that plays a role in the non-canonical pairing of leukotoxins in *S. aureus* pathogenesis. HlgA dominated the hemolytic activity when co-expressed with Hla. Thus, a combination of HlgA and Hla as vaccine components could be therapeutically targeted to inhibit hemolytic activity in humans [22]. Based on these findings, we designed a combination vaccine using four pore-forming toxoids.

Recently, some studies reported that T helper (Th)1 and Th17 CMI plays a key role in resistance to *S. aureus* infections. A vaccine targeting *S. aureus*-induced pneumonia significantly reduced bacterial load, mediated by interferon-γ (IFN-γ) and interleukin-17 (IL-17) produced by CD4+ T cells [16,17]. Additionally, a subset of innate immune lymphocytes plays a critical role in host mucosal defenses against *S. aureus* infection by regulating the initial immune response to lung and skin pathogens through the recruitment of neutrophils, dendritic cells, and macrophages [23,24]. It is well known that vaccine-induced immune responses are strongly influenced by adjuvant selection. Many studies have been conducted to identify suitable adjuvants that enhance CD4 and CD8 cell functions related to the production of IFN-γ and IL-17, which recruit neutrophils, macrophages, and dendritic cells to infection sites for bacterial clearance [25-27]. Several adjuvants that activate the innate immune system for host mucosal defenses and generate cytokine responses have been tested in MRSA vaccine development [28]. These studies underscore the importance of adjuvant selection in *S. aureus* vaccine development and suggest that alternatives to alum may be required.

Based on this background, we conducted this study using two types of β-glucan as alternatives to alum adjuvant. Beta-glucan is well known as a potent inducer of innate immune cell reprogramming and is linked to T-cell activation [29-31]. In this study, we used two types of β-glucan: one purified from synthetic fermented oats with lactobacilli and bifidobacteria, and the other purified from raw oats. These raw materials were provided by With-BioCosPharm in South Korea. The results of this study showed that humoral immune responses increased progressively with vaccination in all study groups, with the highest levels of LukS and Hla_{H35L} IgG observed in group 1 (Fig. 1). All rabbits in group 1 survived and exhibited very mild weight changes after challenges (Fig. 2). Additionally, kidney abscess formation was significantly reduced (no abscess formation in 60% of cases), and CFU levels were markedly decreased in group 1, which received the β-glucan purified from synthetic fermented oats (Figs. 3, 4). In contrast, all cases in group 3, which received β-glucan purified from raw oats, exhibited kidney abscess formation and higher CFU levels (Figs. 3, 4). These results suggest that the β-glucan purified from synthetic fermented oats may have intracellular opsonic phagocytic effects via CMI responses. We hypothesize that the structure of the β-glucan may have been effectively modified through the fermentation process to activate innate immune cells and bind recombinant vaccine antigens. Notably, the amylose level in the β-glucan purified from fermented oats was significantly lower than that in the β-glucan purified from raw oats (unpublished data). This structural change may render it more suitable for antigen presentation and immune cell activation, though further studies are needed to elucidate the mode of action.

Additionally, there were no deaths, and kidney abscess formation was reduced (40% of cases), with low total CFU observed in the alum salt adjuvant group (Figs. 3–5). These results represent an improvement compared to a previous study that did not show reduced renal abscess cases [13]. We attribute these improvements to the modified immunization schedule and vaccine toxoid component dosage. Based on these findings, the four pore-forming toxoid combination vaccine may protect rabbits from the lethal effects of staphy-

lococcal systemic infection by neutralizing staphylococcal toxins and preventing the dissemination and abscess formation of *S. aureus*.

While this study provides valuable insights into the efficacy of a quadrivalent recombinant pore-forming toxoid vaccine in rabbits, several limitations should be noted. First, the study was conducted using a small sample size, which may limit the generalizability of the findings. Additionally, the use of outbred New Zealand White rabbits may not fully represent the complex immune responses observed in humans. Another limitation is the focus on a single strain of *S. aureus* (USA300 LAC), which may not capture the diversity of virulence factors present in other clinically relevant strains. Future studies should address these limitations by including larger, more diverse populations to better understand the vaccine's protective effects and potential for clinical application.

In conclusion, based on the results of our study, we conclude that the quadrivalent recombinant pore-forming toxoid vaccine demonstrates strong protective immune properties, as evidenced by the survival of all study rabbits and the absence of serious adverse effects after immunizations and challenges. Furthermore, renal abscess formation was reduced in cases that received β-glucan and alum-containing vaccinations. Additionally, purified oat β-glucan via fermentation exhibited marked inhibitory effects on intracellular MRSA colonization, showing greater effectiveness than alum. This suggests that β-glucan may offer the dual benefit of delivering relevant antigens while providing an effective adjuvant function. As a result, β-glucan could be a promising candidate for inclusion in an MRSA vaccine for future clinical application against *S. aureus*-induced infections. Given the effective results of β-glucan in this study, it should be considered as a suitable adjuvant in the development of MRSA vaccines. However, further research is needed to determine the optimal dose of recombinant pore-forming toxoid antigens and to better understand the detailed mechanisms of β-glucan action.

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