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Bacteriology

Shiga toxin 2eB-transgenic lettuce vaccine: *N*-glycosylation is important for protecting against porcine edema disease

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ABSTRACT. Porcine edema disease (ED) is a life-threatening toxemia caused by enteric infection with Shiga toxin 2e (Stx2e)-producing *Escherichia coli* (STEC) in weaned piglets. We previously reported that the *stx2eB*-transgenic lettuce 2BH strain shows potential for use as an oral vaccine candidate against ED. However, the 2BH strain expressed a hemagglutinin (HA)-tag together with Stx2eB and contained non-canonical *N*-glycosylation. Therefore, we developed two Stx2eB-lettuce strains, the 3 (G+) strain in which the HA-tag was removed from 2BH, and the 3 (G-) lettuce strain, in which the 73rd Asn was replaced with Ser to prevent non-canonical *N*-glycosylation of Stx2eB from the 3 (G+) strain. We examined the protective effect of these newly developed two strains compared with the previous 2BH strain against ED using a colostrum-deprived piglet STEC infection model. We found that the *N*-glycosylated 2BH and 3 (G+) strains relieved the pathogenic symptoms of ED in STEC-challenged piglets, whereas the non-glycosylated 3 (G-) strain did not. *N*-Glycosylation of the Stx2eB product in lettuce may be involved in the immune response in piglets.

KEY WORDS: colostrum-deprived piglet infection model, glycosylation, porcine edema disease, Shiga toxin 2e-producing *Escherichia coli*, *stx2eB*-transgenic lettuce vaccine

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Transgenic plant-based oral vaccines have been of interest as "new generation vaccines" against human and animal infectious diseases because of their several advantages compared to classical injection vaccines: 1) oral vaccines induce both the mucosal and systemic immune systems [13]; 2) plant cells protect antigens from acidic or enzymatic degradation in the stomach and release the antigen in the intestine following plant cell wall digestion by the gut microflora [2]; 3) plant products can typically be stored for long periods at room temperature [17]; and 4) leafy plants such as lettuce grow rapidly and are suitable for production in closed plant factory systems; therefore, the cultivation conditions can be optimized to achieve the maximum yield of the vaccine antigen and control the quality of plants to produce high-quality medical products [18].

Our research has focused on developing transgenic lettuce plants as candidate vaccines against porcine edema disease (ED) [16]. ED is a toxemia that occurs among weaned piglets and is caused by enteric infection with Shiga toxin 2e (Stx2e)-producing *Escherichia coli* (STEC). Major symptoms of ED include edema, emaciation, neurological disorders such as ataxia or paralysis, and death in severe cases [9]. The B subunit of Stx2e (Stx2eB) is responsible for binding to the host receptor globotetraosylceramide Gb4 [3]; therefore, in our previous study, we introduced a tandem-*stx2eB* transgene into a lettuce plant [14]. We showed that oral administration of Stx2eB-transgenic lettuce relieved ED symptoms in STEC-challenged piglets [7].

However, the previous vaccine lettuce contained a hemagglutinin (HA)-tag as a marker for quantifying Stx2eB production in lettuce. After developing a method for quantifying Stx2eB [14], the HA-tag was unnecessary; therefore, to simplify the vaccine product, we removed the HA-tag from the *stx2eB* transgene. Additionally, as Stx2eB produced in plant cells was *N*-glycosylated non-canonically [15], we modified the *N*-glycosylated amino acid residues in the transgene to obtain glycosylation-free Stx2eB vaccine and assessed how glycosylation affected the antigenicity of Stx2eB in the vaccine lettuce.

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In this study, we compared the ability of the three Stx2eB-lettuces, i.e., previously constructed 2BH strain, HA-tag-free and *N*-glycosylated 3 (G+) strain, and HA-tag-free and *N*-glycosylation-free 3 (G-) strain, to protect against ED using a colostrum-deprived piglet STEC infection model [19].

MATERIALS AND METHODS

Lettuce strains

The transgene of Stx2eB-lettuce strain 2BH [7] was used as a parental template to construct transgenes in newly developed lettuce strains 3 (G+) and 3 (G-) (Fig. 1). Briefly, the HA-tag (Tyr-Pro-Tyr-Asp-Tyr-Pro-Asp-Tyr-Ala) was removed from the 2BH transgene, resulting in 3 (G+) transgene. Next, the 73Asn-Thr-Cys in Stx2eB (non-canonical *N*-glycosylation sites [15]) and potential *N*-glycosylation sites Asn-Arg-Ser at the junction of Stx2eB and the linker were substituted by Ser-Thr-Cys and Asn-Arg-Ala, respectively, to produce the 3 (G-) transgene. These constructs were introduced into lettuce plants through *Agrobacterium*-mediated transformation, as previously described [16]. A lettuce plant transformed with the empty vector pRI909 [20] was used as a control vaccine as previously described [7]. Production of these genetically modified organisms was approved by the Idemitsu Kosan Co., Ltd., Advanced Technology Research Laboratories Safety Committee for Recombinant DNA Experiments (approval numbers 2008-01, 2009-01, 2010-01, and 2011-01).

All lettuce leaves were freeze-dried, ground in a mill (Wonder Blender WB-1; Osaka Chemical, Osaka, Japan), and stored at −80°C until use. The Stx2eB content in each lot of lettuce plants was determined using dot-blot analysis and expressed as mg/g powdered lettuce dry weight, and the expressed Stx2eB product was analyzed by western blotting, as described previously [14]. Lettuce powder was reconstituted in warm water and administered using gastric tubes (Jfeed Nutrition Catheter JF-C15120Q; JMS, Hiroshima, Japan).

STEC strain

The ED-derived STEC strain KY010 (serotype O139:H1, *stx2e*$^{+}$, *fadA*$^{+}$, *elt*$^{-}$, *est*$^{-}$) [1] was used for oral challenge. The STEC sample was prepared and administered as previously described [24]. Briefly, the STEC strain was cultured in trypticase soy broth at 37°C for 4 hr, centrifuged at 3,000 × *g* for 15 min, and suspended in saline. Chitosan-coated capsules (Sansyoiyaku, Tokyo, Japan) were filled with approximately 10^{11} colony-forming units of STEC as one dose and then orally administered to vaccinated or control piglets from day 25 for three consecutive days [day post-infection (dpi) 0, dpi1, and dpi2]. The same types of capsules filled with saline were administered as a non-challenge control.

Pigs and diets

Experiments 1 and 2 were performed in accordance with the guidelines for animal studies at the KYODOKEN Institute (Kyoto, Japan) and approved by the Experimental Animal Committee of KYODOKEN Institute (approval numbers EB104062 and

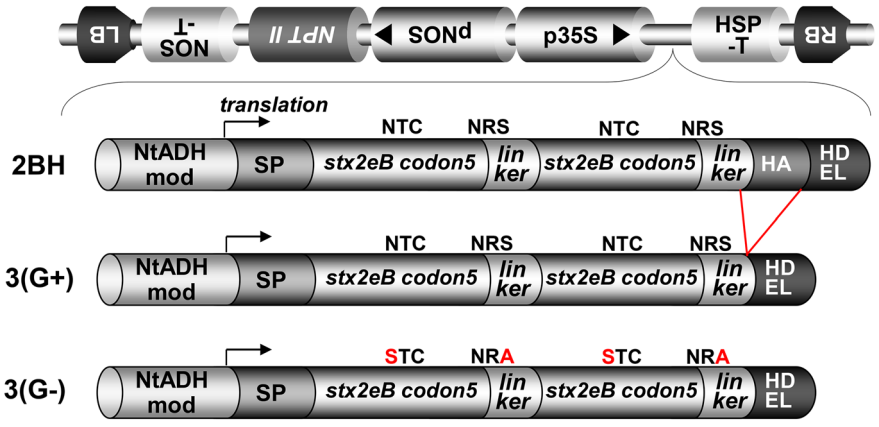

Fig. 1. Shiga toxin 2eB (Stx2eB) transgene constructs. The modifications of the transgene in this study are indicated by red lines (deletion of hemagglutinin-tag from 2BH) and red characters [substitutions of amino acid residues in 3 (G-)]. Codon-modified *stx2eB* (*stx2eB* codon 5) was duplicated and fused with a translational enhancer *NtADH* 5′-untranslated region [20], in which three nucleotides immediately upstream of the AUG codon were changed to AAG [22] (NtADH mod), for high-level Stx2eB expression in lettuce plants. The tandem Stx2eB protein and linker (PG12; Arg-Ser-Pro-Gly-Ser-Gly-Pro-Gly-Ser-Pro-Arg-Ser) were translated together with the N-terminal secretion signal peptide (SP) and C-terminal endoplasmic reticulum (ER) retention signal (His-Asp-Glu-Leu, HDEL) for stable accumulation in the ER [16]. Expression cassettes for NPTII and the vaccine antigen were arranged in a head-to-head orientation in a pRI909 plasmid. NOS-T, transcription terminator from *Agrobacterium tumefaciens* nopaline synthase gene; NPTII, neomycin phosphotransferase gene for kanamycin resistance, derived from *E. coli*; pNOS, *A. tumefaciens* nopaline synthase gene promoter; p35S, cauliflower mosaic virus 35S RNA promoter; HSP-T, transcription terminator from *Arabidopsis thaliana* heat shock protein 18.2 gene; RB, right border; LB, left border.

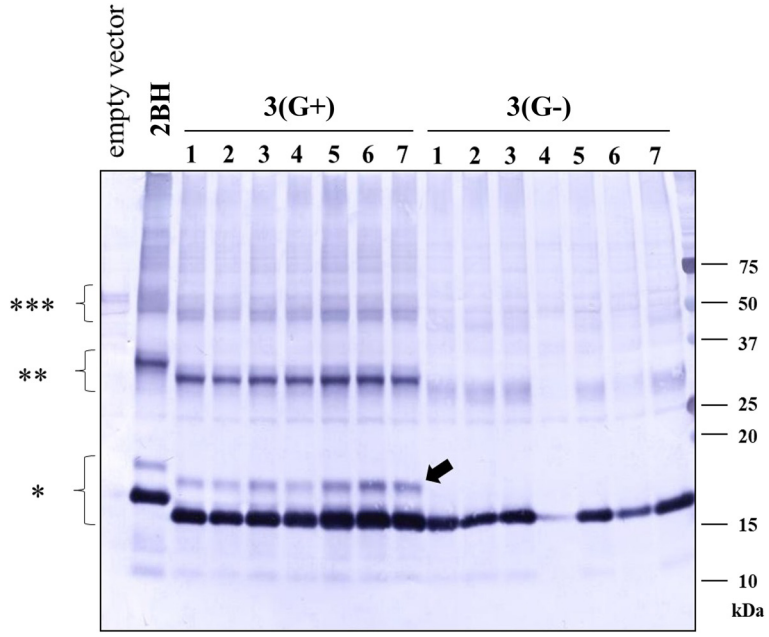

Fig. 2. Shiga toxin 2eB (Stx2eB) expression in transgenic lettuce plants. Total soluble protein was extracted from transgenic lettuce plants and analyzed using western blotting as previously described [14]. Seven independent lines from 3 (G+) and 3 (G-) were analyzed; the amount of total soluble protein loaded in each lane was 3.75 µg. Mouse antiserum against KLH-conjugated peptide 22b-35 (Ala-Asp-Cys-Ala-Lys-Gly-Lys-Ile-Glu-Phe-Ser-Lys-Tyr-Asn corresponding to amino acids 22–35 of Stx2eB) was used as the primary antibody. Several bands corresponding to monomers (*), dimers (**), and trimers (***) were detected. For the 3 (G+) and 3 (G-) strains, the molecular weights corresponding to the signals were lower than that of the 2BH strain, indicating the absence of the hemagglutinin-tag. Partially *N*-glycosylated Stx2eB bands were detected in 2BH and 3 (G+) strains (arrow).

EB104078, respectively). In each experiment, two healthy pregnant sows (Landrace × Large White) impregnated by Duroc boars were transferred from a commercial pig farm to fallowing pens at KYODOKEN Institute at eight days before delivery. The sows were fed a commercial diet (Bree-Meal Maxim, Feed One, Yokohama, Japan) throughout the study. After delivery, neonatal piglets were immediately separated from their sows and fed artificial milk (Meiji Hohoemi, Meiji, Tokyo, Japan) rather than colostrum for 24 hr at 2–3 hr intervals [19]. The piglets were then returned to their own sow's stall for nursing and kept breastfed naturally until weaning (21-days-old) (Fig. 3).

Lettuce administration, challenge, and observation

The piglets were divided at 6 days of age into seven and four groups in experiments 1 and 2, respectively, to avoid weight-, gender-, or sow-based bias and orally administered lettuce at 6, 12, and 18 days of age. All 21-day-old piglets were weaned and transferred to concrete pens with brooders at the KYODOKEN Institute for each experimental group and fed a commercial diet (SDS No. 1; Feed-One) *ad libitum*. All piglets were in good health and negative for *stx2e* genes according to quantitative PCR of the feces. At 25 days of age (dpi0), oral challenge with virulent STEC for three consecutive days was performed in the piglets as described above. The grouping of the piglets, vaccination, and challenge settings in experiments 1 and 2 are summarized in Tables 1 and 2, respectively.

After oral challenge with virulent STEC, clinical symptoms such as vitality, appetite, respiration, feces, palpebral edema, neurologic impairment, and lateral recumbency of individual piglets were evaluated daily and scored as previously described [24]. Body weight and food intake were measured, and fecal and blood samples were collected on the days indicated in Fig. 3. The feed conversion ratio (FCR) was calculated by food intake (kg) per body weight gain (kg) from dpi0 to dpi9. All piglets were euthanized at dpi9 using exsanguination under anesthesia with an overdose of sodium pentobarbital (Somnopentyl, Kyoritsu Seiyaku, Tokyo, Japan). After ventral midline incision, the digestive tract and other organs were evaluated to detect gross abnormalities. The luminal contents were collected from the ileum and cecum. The jejunum, ileum, terminal ileum, eyelids, lungs, kidneys, liver, spleen, mesenteric lymph nodes, and other organs showing abnormalities were collected and fixed in 10% (v/v) phosphate-buffered formalin solution. The intestine and organs were evaluated in histopathological analysis as previously described [24]. The abundance of STEC in the feces was estimated on dpi3 using quantitative PCR of *stx2e* as previously described [23].

Statistical analyses

All values are shown as the means ± standard deviation except for groups 1F and 1G in experiment 1, which contained two piglets each (Table 1); thus, only the means are presented without the statistical analyses described below. The differences

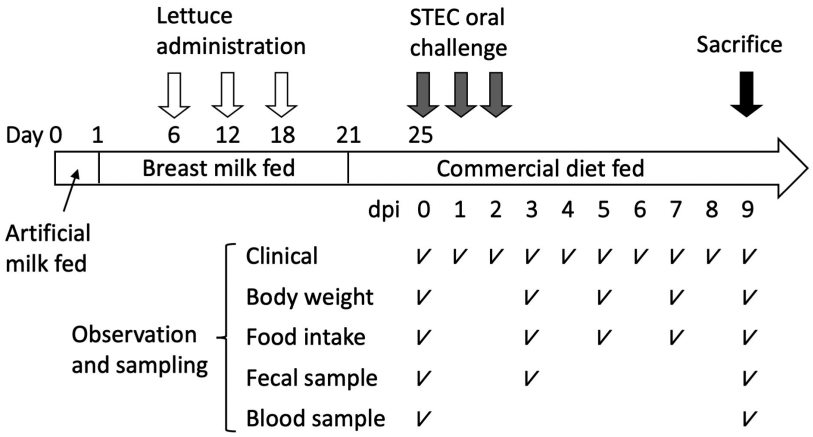

Fig. 3. Schematic representation of experiments in this study. The lifetime course of the piglets is shown as the age in days as a horizontal thick arrow from left to right; the diets provided are indicated. The days of lettuce administration and Shiga toxin 2e-producing *Escherichia coli* (STEC) oral challenge are indicated as open and gray vertical arrows, respectively. The day of sacrifice is indicated by a filled vertical arrow. From the first day of STEC challenge (25 days), days post-infection (dpi) is indicated below the horizontal arrow until the day of sacrifice at dpi9. Observations and sample collection were performed on the indicated days (*V*).

Table 1. Summary of vaccination and challenge settings in experiment 1

Group	n	Vaccine lettuce			Oral challege
		Strain	Dose (g DW)	Stx2eB content (mg/dose)	
1A	3	vector	0.85	NA	STEC
1B	3	3 (G-)	0.85	2.3	STEC
1C	3	2BH	0.5	2.3	STEC
1D	3	2BH	0.05	0.23	STEC
1E	3	2BH	0.005	0.023	STEC
1F	2	NA	NA	NA	Saline
1G	2	NA	NA	NA	STEC

DW, dry weight; Stx2eB, Shiga toxin 2eB; STEC, Shiga toxin 2e-producing *Escherichia coli*; NA, not applicable.

Table 2. Summary of vaccination and challenge settings in experiment 2

Group	n	Vaccine lettuce			Oral challege
		Strain	Dose (g DW)	Stx2eB content (mg/dose)	
2A	5	vector	0.67	NA	STEC
2B	5	3 (G+)	0.67	2.3	STEC
2C	5	3 (G+)	0.34	1.2	STEC
2D	3	NA	NA	NA	Saline

DW, dry weight; Stx2eB, Shiga toxin 2eB; STEC, Shiga toxin 2e-producing *Escherichia coli*; NA, not applicable.

between groups in each experiment for body weight gain and bacterial count were analyzed using one-way analysis of variance or Kruskal-Wallis test after evaluating the homoscedasticity of the data using Bartlett's test. When significant differences were detected, parametric or non-parametric Tukey-Kramer *post hoc* test was applied to compare differences among groups in each experiment. Clinical and pathological scores were analyzed using the Kruskal-Wallis test, and a non-parametric Tukey-Kramer *post hoc* comparison was performed when significance differences were detected between groups. Differences among means were considered as significant at $P<0.05$ and tended to be significant at $P<0.1$. All data were analyzed using STATCEL (OMS, Saitama, Japan), an add-on application for Microsoft Excel (Seattle, WA, USA).

RESULTS

Construction of stx2eB-transgenic lettuce strains

A schematic representation of the previous and new *stx2eB* transgenes is shown in Fig. 1. As described above, the HA-tag was removed from the 2BH strain, resulting in the 3 (G+) strain. The *N*-glycosylation target sites were further substituted to produce non-glycosylated Stx2eB in the 3 (G-) strain. After transforming these constructs into lettuce plants, we evaluated the production of Stx2eB in several transformant lines using western blot analysis (Fig. 2). The newly constructed lettuce strains 3 (G+) and 3 (G-) produced a tandem Stx2eB product with a molecular mass that was 1.1 kDa (corresponding to the size of the HA-tag) lower than that of Stx2eB product of the original 2BH strain. As expected, glycosylation of Stx2eB was completely suppressed in the 3 (G-) strain compared to the 3 (G+) strain.

Experiment 1

From dpi0 to dpi9, the gain in body weight in groups 1A and 1B was significantly lower than that in groups 1C and 1D

(P<0.01) (Fig. 4A). The FCR of group 1B was almost the same as that of the vaccine-untreated challenged group 1G, whereas those of groups 1C, 1D, and 1E were as low as that of the unchallenged group 1F (Fig. 4B). The total clinical scores of groups 1A and 1B were significantly higher than those of groups 1C (P=0.02) and 1D (P=0.02), respectively (Fig. 4C). Additionally, for individual clinical scores, groups 1A and 1B showed comparable scores as the vaccine-untreated and STEC-challenged group 1G, whereas those of groups 1C, 1D, and 1E were similar to the score of the vaccine-untreated and STEC-unchallenged group 1F (Supplementary Table 1). All three piglets in group 1E showed slight infiltration of eosinophils in the palpebral tissues (Supplementary Fig. 1A), suggesting that 0.005 g per dose is insufficient to fully protect against artificial ED. Other pathological features observed included interstitial pneumonia or epithelial congestion in the colon (Supplementary Fig. 1B and 1C) in some piglets in each group except for group 1F, although the differences among groups were not significant (data not shown).

Experiment 2

Although not significant, the body weight gain of piglets vaccinated with Stx2eB-lettuce (groups 2B and 2C) was better than that of unvaccinated group 2A from dpi0 to dpi9 (Fig. 5A). The effect was more apparent for the FCR. The FCR values for groups 2B and 2C were as low as that in the unchallenged control group 2D (Fig. 5B). The total clinical score of group 2A was significantly higher than those of groups 2B and 2C (P=0.03) (Fig. 5C). Regarding the individual clinical symptoms, particularly palpebral edema and neurological impairment, which are the most characteristic clinical symptoms of ED, were more severe in the unvaccinated group 2A than in the other groups (Supplementary Table 2). These results suggest that the 3 (G+) lettuce strain, containing at least 1.2 mg of Stx2eB per dose, effectively decreased ED symptoms. Although there were slight pathological observations in some piglets, including in the control group 2D, there were no significant differences among groups (data not shown).

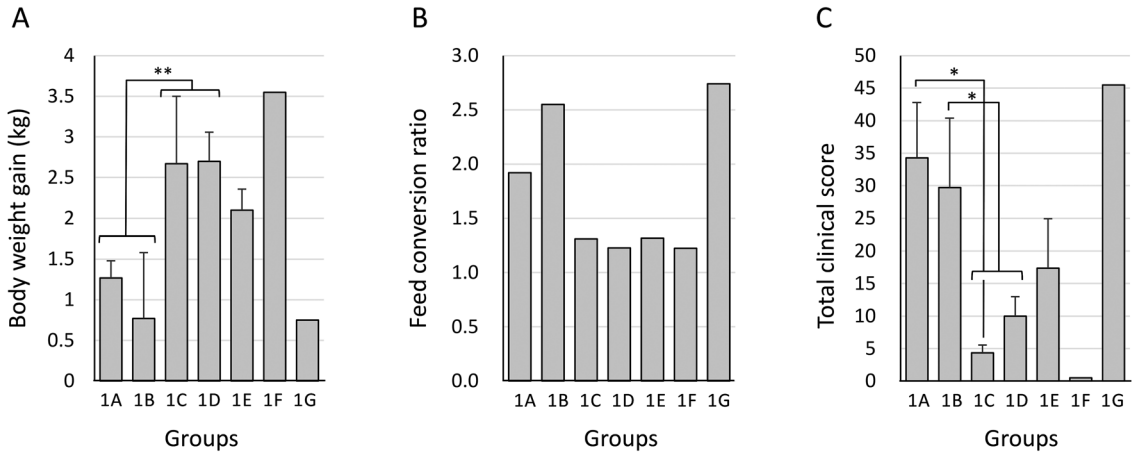

Fig. 4. Body weight gain **(A)**, feed conversion ratio **(B)**, and total clinical score **(C)** in experiment 1. Table 1 and the text describe each group. Error bars in panels A and C represent standard deviations. Groups 1F and 1G were excluded from statistical analysis because they contained only two piglets. The scores of each clinical parameter in panel C are listed in Supplementary Table 1. **; P<0.01. *; P<0.05.

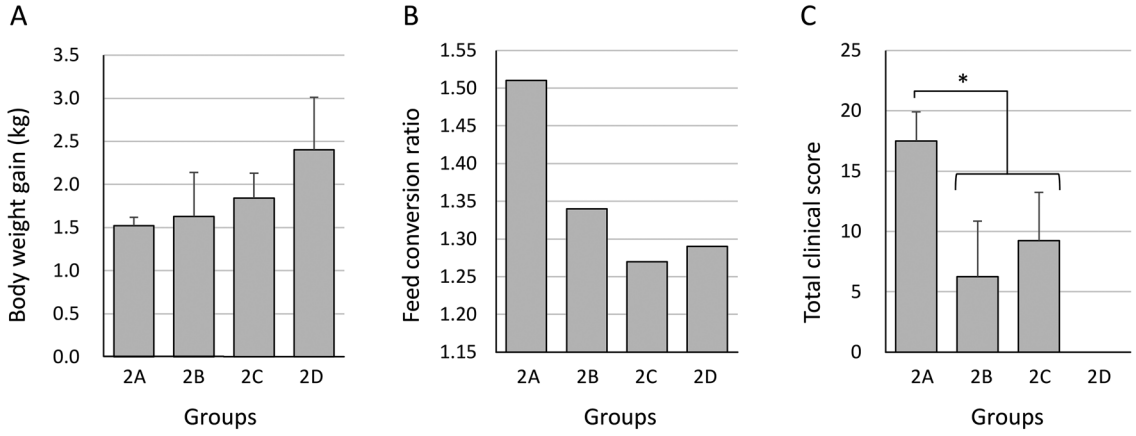

Fig. 5. Body weight gain **(A)**, feed conversion ratio **(B)**, and total clinical score **(C)** in experiment 2. Refer to Table 2 and the text for information on each group. Error bars in panels A and C indicate the standard deviations. The scores for each clinical parameter in panel C are listed in Supplementary Table 2. *; P<0.05.

In both experiments 1 and 2, oral vaccination of Stx2eB-lettuce did not reduce the challenged STEC count in the feces on dpi3 (Supplementary Table 3).

DISCUSSION

The efficacy of lettuce strain 2BH in experiment 1 and strain 3 (G+) in experiment 2 in reducing the ED pathogenic symptoms appeared to be comparable, suggesting that the presence of the HA-tag in the transgene was not related to the vaccine effect. Additionally, sufficient protective effects against ED were observed when the dose of Stx2eB was reduced to 0.23 mg (group 1D) or 1.2 mg (group 2C). Although the minimal effective amount should be determined, it is expected to be much lower than the previously assumed level (2.3 mg) [11].

In contrast, lettuce strain 3 (G-), which expressed non-glycosylated Stx2eB, did not protect the STEC-challenged piglets in group 1B from ED symptoms in experiment 1. The body weight gain, FCR, and total clinical scores of group 1B were similar to those in the unvaccinated and STEC-infected group 1G (Fig. 4). This result suggests that *N*-glycosylation at 73Asn of Stx2eB in lettuce strains 2BH and 3 (G+) enhanced the protective effect against ED. This may be because non-canonical *N*-glycosylation of Stx2eB produced in lettuce cells produces high molecular stability. Artificial or non-canonical glycosylation has been reported to improve the thermostability [5] and pH stability and protease-resistance of proteins [8], and many human pharmaceutical proteins have been established based on this property [21]. The molecular stability of glycosylated and non-glycosylated Stx2eB (produced in lettuce strains 3 (G+) and 3 (G-), respectively) against acidic or alkaline pH and digestive proteinase should be further examined. Another possibility is that such glycosylation induces enhanced immunoreaction against protein antigens [4, 10]. C-type lectin receptors on dendritic cells were reported to recognize glycosylation patterns that are distinct from those of host carbohydrates as pathogens. Moreover, these receptors promote intracellular signaling to induce nuclear factor-κB activation, which plays a critical role in innate immunity and inflammatory responses [12] and helper T cell differentiation, particularly of Th1 and Th17 cells [6]. In agreement with the results of our previous study [7], administration of Stx2eB-lettuce did not lead to Stx2e-specific antibody responses (Supplementary Fig. 2). All fecal anti-Stx2e IgA levels were below the detection limit (data not shown). In addition, we observed that Stx2eB expressed in *E. coli* stimulated murine macrophages to induce the inflammatory cytokine tumor necrosis factor α (Supplementary Fig. 3). Further, *Salmonella* Enteritidis infection experiments using chicks showed that Stx2eB-lettuce administration significantly improved body weight gain at dpi3, and *Salmonella* contents decreased by half (6.77 vs 7.03 log cells/g; no significant difference) in the cecum at dpi7 (Supplementary Fig. 4). Taken together, the intrinsic nature of Stx2eB as an oral antigen induces non-humoral immunity, such as a Th1 or inflammatory response, and non-canonical *N*-glycosylation of Stx2eB in plant cells may enhance such *in vivo* responses in piglets.

CONFLICT OF INTERESTS. The authors declare that there is no conflict of interests.

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