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Effects of *Limosilactobacillus reuteri* Strain 164 on Intestinal Gene Expression and Colon Inflammation: Potential as a Probiotic Strain

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Abstract

As weaning piglets are easily susceptible to infections and diarrhea, addressing these issues is critical in pig production. Therefore, the use of probiotics has garnered increasing attention. *Limosilactobacillus reuteri* (*L. reuteri*) is one of the most studied probiotics that can colonize the gut of a wide range of animals. Studies examining the effects of *L. reuteri* in pigs have suggested that it can be used as a probiotic to reduce intestinal diseases in piglets and improve productivity. In our study, *L. reuteri* strain 164 (L164) was isolated from farm pig feces. The isolated L164 was evaluated for antibiotic susceptibility, and we found that L164 had little antibiotic resistance. L164 is also resistant to low pH and bile treatment, similar to *Lacticaseibacillus rhamnosus* strain GG, a notable probiotic strain. The gene expression analysis of L164-ingested mice indicated that L164 may activate the aryl hydrocarbon receptor, decrease intestinal epithelial stem cells in the ileum, and increase enterochromaffin cells in the colon. Furthermore, experiments using a mouse model of colitis showed that L164 inhibited colon shortening caused by a chemical colitogen and reduced diarrheal symptoms. These results suggest that L164 is a promising probiotic for weaning piglets.

Discipline: Animal Science
Additional key words: antibiotics, weaning piglets

Introduction

Early weaning is commonly practiced on pig farms to increase the production yield. This period is critical for pig production as weaning piglets are easily susceptible to infectious diseases and diarrhea. However, the use of antibiotics is currently limited in pig farms to reduce the risk of pathogens acquiring antibiotic resistance. Great efforts have been made to develop strategies to overcome the issues of weaning piglets and replacing the use of antibiotics. Therefore, the use of probiotics has recently garnered considerable attention (Barba-Vidal et al. 2018).

Limosilactobacillus reuteri is one of the most studied probiotics, with the ability to colonize a wide range of animals (Abuqwider et al. 2022). Growing evidence has suggested that *L. reuteri* exerts protective effects on the intestinal tract (Anjum et al. 2023). Several studies have examined the effects of *L. reuteri* on experimental colitis and reported its protective effects when administered alone (Diez-Echave et al. 2021, Liu et al. 2022, Seo et al. 2021, Sun et al. 2018, Wang et al. 2020a), in combination with other bacteria (Wang et al. 2020b, Xu et al. 2022), or combination with prebiotics (Lee et al. 2022). Furthermore, several mechanisms of intestinal protection by *L. reuteri* have been investigated, including the activation of the aryl hydrocarbon receptor (AhR) and induction of interleukin (IL)-22 (Zelante et al. 2013), suppression of inflammatory cytokines, such as IL-1β, IL-6, tumor necrosis factor (TNF)-α (Diez-Echave et al. 2021, Sun et al. 2018, Wang et al. 2020a), and

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enhancement of the expression of tight junction-related proteins, such as occludin and ZO-1 (Seo et al. 2021). The use of *L. reuteri* in weaning piglets has also been investigated; studies have found that feed fermented with *L. reuteri* reduces the level of colonization of enterotoxigenic *Escherichia coli* (Yang et al. 2015) and that the ingestion of *L. reuteri* decreases the incidence of diarrhea (Dell'Anno et al. 2021). Additionally, the administration of *L. reuteri* for 175 days after weaning has been found to improve the meat quality of pigs compared with that of the antibiotic-treated group (Tian et al. 2021). Thus, *L. reuteri* can be used as a probiotic to reduce intestinal diseases in weaning piglets and improve productivity.

In this study, we isolated the *L. reuteri* strain 164 (L164) from farm pig feces to obtain a probiotic strain that could be a promising alternative to antibiotics for weaning piglets. Isolated L164 was evaluated for antibiotic susceptibility to ensure that the strain was not at risk of transmitting antibiotic resistance in anticipation of its use on farms. Additionally, we evaluated its potential as a probiotic. To assess the survival potential of this strain during its passage through the digestive tract, we measured its resistance to low pH and bile treatment. Furthermore, as a preliminary step to piglet administration, we evaluated the effects of L164 ingestion using mouse models for efficient confirmation of the effects of the administered bacteria, and we have established analytical tools and evaluation systems for mouse-based studies. In the present study, we used a normal mouse model to evaluate the effects of L164 ingestion on the intestinal epithelium of the host and a murine colitis model to evaluate its protective effect on the intestinal tract.

Materials and methods

1. Bacteria

L164 was isolated from the feces of a Large White sow after farrowing (second parity) on a de Man-Rogosa-Sharpe (MRS) agar plate and identified using a MALDI Biotyper (Bruker, Billerica, MA, USA). The *Lacticaseibacillus rhamnosus* strain GG (LGG), which is used as a probiotic strain (Capurso 2019) worldwide, was used as the control to evaluate the resistance to low pH and bile treatment.

2. Antibiotic susceptibility testing of isolated L164

L164 cells were cultured for 24 h in MRS medium, and the culture suspension was plated on an MRS agar plate. The BD Sensi Disc (BD, Tokyo, Japan) was placed on the MRS agar plates. After culturing at 37℃ for 48 h, antibiotic susceptibility was evaluated by measuring the inhibition zone. BD Sensi Discs of ampicillin, erythromycin, tetracycline, chloramphenicol, gentamicin, streptomycin, cefazolin, and the combination of trimethoprim and sulfamethoxazole (TMP-SMX) were used.

3. Evaluation of resistance to low pH and bile treatment

The bacterial cells were treated with low pH in accordance with the method of Berrada et al. (1991) and subsequently treated with bile in accordance with the method of Kimoto et al. (2002). In particular, after overnight culturing, the bacterial cells of L164 and LGG were harvested by centrifugation, resuspended in 0.85% NaCl, with a pH of 2.5 adjusted with 1 N HCl, and held at 37℃ for 90 min. The cells were centrifuged and resuspended in 50 mM sodium phosphate buffer (pH 7.0). Subsequently, oxgall solution was added to the cell suspension (final concentration of 0.3% (v/v)), and the mixtures were incubated in a water bath at 37°C. Samples were collected at 0 and 3 h and plated on MRS agar plates. The colonies were counted after overnight incubation at 37°C.

4. Mice

Female C57BL/6J mice were purchased from Charles River Japan (Kanagawa, Japan); they were fed standard chow (MF chow, Oriental Yeast, Tokyo, Japan) and water ad libitum under constant temperature (24℃) and humidity (45%), and a 12-h light/dark cycle. Three animals were housed per cage and allowed to acclimatize for at least two weeks. All animal experiments were conducted in accordance with the Animal Experiment Guidelines of the National Agriculture and Food Research Organization and were approved by the Animal Experiment Committee (approval number: 21D102ILGS, R4-M03-NILGS).

5. Preparation of L164 supplemented diet

L164 cells were cultured overnight in an MRS medium, and the bacterial cells were harvested (5,000 ×g, 10 min) and washed with saline. The cells were then suspended in cryoprotectant (10% skim milk, 5% trehalose, and 1.5% sodium glutamate solution) and freeze-dried. The prepared powder was added to the MF chow at a concentration of 1% (containing L164, 2×10^8 cfu/g). MF chow supplemented with a 1% protective agent without bacterial cells was used as the control diet.

6. Evaluation of the effect of ingestion of L164 on gene expression in mouse ileum and colon

Two groups of 8-week-old female C57BL/6j mice (n = 6) were established: the control (CONT) and L164 groups. The CONT and L164 groups were fed the control and L164-supplemented diets, respectively, for nine days. The mice were euthanized by cervical dislocation, and the ileum and colon were collected for real-time polymerase chain reaction (PCR).

7. Dextran sulfate sodium (DSS)-induced colitis

Dextran sulfate sodium (DSS) colitis was induced in mice according to the protocol described by Chassaing et al. (2014). Two groups of 8-week-old female C57BL/6j mice (n = 9) were employed as the DSS and L164/DSS groups. The DSS and L164/DSS groups were fed the control and L164-supplemented diets ad libitum, respectively. After one week, their drinking water was replaced with 2% (w/v) DSS water, and the mice were allowed to drink DSS water for five days to induce diarrhea. The DSS water was replaced with normal water, and the mice were maintained for two days. The body weights were measured daily, and diarrhea scores were evaluated on the fifth and seventh days after the start of DSS administration. The diarrhea score was evaluated as previously described (Aoki et al. 2018); normal stools: 0, loose stools: 1, diarrheal stools: 2, and watery stools: 3. After the mice were euthanized by cervical dislocation, the colons were collected. As DSS administration is known to impair the mouse colon and shorten its length (Chassaing et al. 2014, Eichele & Kharbanda 2017), the colon lengths were measured. The colon samples were further subjected to real-time PCR and histological analyses.

8. Real-time PCR analysis

The tissues were treated with RNAlater (Thermo Fisher Scientific). Total RNA was extracted using RNeasy Mini Kit (Qiagen, Hilden, Germany), and 0.5 μg of RNA was converted into cDNA using ReverTra Ace qPCR RT Master Mix (Toyobo, Osaka, Japan). Real-time PCR was conducted using the Thunderbird SYBR qPCR Mix (Toyobo) with a C1000 Thermal Cycler (BIORAD, Hercules, CA, USA). The analyzed genes and their corresponding primer sequences are listed in Table 1. PCR was conducted at 95°C for 60 s for initial denaturation, followed by 40 cycles of denaturation (95°C, 15 s), annealing, and elongation (60°C, 30 s). The expression of each gene was normalized by β-actin gene expression.

9. Microscopic and histological analysis

The colons were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for at least 24 h. They were then embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E). The H&E-stained sections were observed under an AE2000 microscope (Shimadzu, Kyoto, Japan). Histological scores were evaluated in accordance with the method of Park et al. (2015).

Table 1. List of primers

Primer name	Primer sequence
Atoh1 forward	ATCCCGTCCTTCAACAACGAC
Atoh1 reverse	CTCTCCGACATTGGGAGTCTG
Car1 forward	GACTGGGGATATGGAAGCGAA
Car1 reverse	TGCAGGATTATAGGAGATGCTGA
Car4 forward	CTCCTTCTTGCTCTGCTG
Car4 reverse	GACTGCTGATTCTCCTTA
ChgA forward	CCAAGGTGATGAAGTGCGTC
ChgA reverse	GGTGTCGCAGGATAGAGAGGA
Cyp1A1 forward	GGTTAACCATGACCGGGAACT
Cyp1A1 reverse	TGCCCAAACCAAAGAGAGTGA
Gcg forward	CATTTACTTTGTGGCTGGATTGC
Gcg reverse	CGGTTCCTCTTGGTGTTCATC
IL-10 forward	ATGCTGCCTGCTCTTACTGACTG
IL-10 reverse	CCCAAGTAACCCTTAAAGTCCTGC
IL-1β forward	GCAACTGTTCCTGAACTCAACT
IL-1β reverse	ATCTTTTGGGGTCCGTCAACT
IL-22 forward	GGCCAGCCTTGCAGATAACA
IL-22 reverse	GCTGATGTGACAGGAGCTGA
IL-6 forward	GAGGATACCACTCCCAACAGACC
IL-6 reverse	AAGTGCATCATCGTTGTTCATACA
Lgr5 forward	CCTACTCGAAGACTTACCCAGT
Lgr5 reverse	GCATTGGGGTGAATGATAGCA
Muc2 forward	AGGGCTCGGAACTCCAGAAA
Muc2 reverse	CCAGGGAATCGGTAGACATCG
Occludin forward	TTGAAAGTCCACCTCCTTACAGA
Occludin reverse	CCGGATAAAAAGAGTACGCTGG
TNF-α reverse	TGGGAGTAGACAAGGTACAACCC
TNF-α forward	CATCTTCTCAAAATTCGAGTGACAA
Tph1 forward	AACAAAGACCATTCCTCCGAAAG
Tph1 reverse	TGTAACAGGCTCACATGATTCTC
ZO-1 forward	GCCGCTAAGAGCACAGCAA
ZO-1 reverse	TCCCCACTCTGAAAATGAGGA
β-actin forward	GGGTCAGAAGGACTCCTATG
β-actin reverse	GTAACAATGCCATGTTCAAT

10. Statistical analysis

Data obtained from real-time PCR analysis and body weight data were analyzed using the Student's t-test. Diarrhea and histological scores were analyzed using the Mann-Whitney U test. Statistical significance was set at $P < 0.05$.

Results

1. Antibiotic susceptibility of L164 isolated from pig feces

Bacteria derived from pig feces are suspected to have antibiotic resistance as they are exposed to antibiotics through feeding or treatment. Therefore, we first evaluated the antibiotic susceptibility of strain L164 and found that it was resistant only to trimethoprim-sulfamethoxazole (TMP-SMX) and susceptible to ampicillin, erythromycin, tetracycline, chloramphenicol, gentamicin, streptomycin, and cefazolin (data not presented).

2. Resistance of L164 to low pH and bile treatment

Notably, probiotics must reach the gastrointestinal tract alive to conduct their functions. Therefore, we evaluated the resistance of L164 cells to low pH and bile treatment. L164 showed a good survival rate, comparable with that of LGG (Table 2).

3. Effect of L164 ingestion on gene expression in mouse ileum and colon

To analyze the effects of L164 ingestion on the intestinal tract, mice were fed a control or L164-supplemented diet for nine days, and gene expression analysis was conducted in the ileum and colon. Analysis of differentiation marker expression in the intestinal epithelium revealed differences in the responses of the ileum and colon to L164 ingestion. In the ileum, Lgr5, namely, a marker of intestinal epithelial stem cells, was significantly suppressed by L164 ingestion, whereas no significant differences were observed in the expressions of Car4, a small intestinal absorptive epithelial marker, Atoh1, an intestinal secretory cell marker, ChgA, an enteroendocrine cell marker, or Muc2, a goblet cell marker (Fig. 1A). In contrast, in the colon, the expressions of Car1 (a marker of colonic absorptive epithelial cells), Atoh1, and ChgA were significantly enhanced by L164 ingestion, whereas no significant differences were observed in the expressions of Muc2 and Lgr5 (Fig. 1B). As the ingestion of L164 significantly enhanced the expression of enteroendocrine cell markers in the colon, we investigated the expressions of Gcg, an L-cell marker, and Tph1, an enterochromaffin cell marker, to determine the type of enteroendocrine cells that were increased. Our results showed that L164 ingestion significantly enhanced Tph1 expression, whereas no significant difference was observed in Gcg expression (Fig. 1B). No differences in Gcg or Tph1 expression were observed in the ileum (Fig. 1A).

L. reuteri exerts beneficial effects on the host by activating AhR in the gastrointestinal tract (Cervantes-Barragan et al. 2017, Natividad et al. 2018, Zelante et al. 2013). To investigate the effect of L164 ingestion on AhR activation in the intestinal tract, we analyzed the expression of Cyp1A1, a sensitive marker of AhR activation. Cyp1A1 expression was significantly enhanced by L164 ingestion in the ileum (Fig. 1A), but no significant difference was observed in the expression of these genes in the colon (Fig. 1B).

Table 2. Resistance of L164 to low pH and bile treatment

Strain	0 h[a]	3 h
L164	8.96 ± 0.11	7.77 ± 0.06
LGG	9.27 ± 0.13	7.79 ± 0.09

[a] The number of viable bacteria is indicated in Log cfu/mL (mean ± SD).

4. Effect of L164 ingestion on the DSS-induced colitis model

Subsequently, we investigated the effects of L164 ingestion on the DSS colitis model. Mice were fed a control (DSS group) or L164 supplemented diet (L164/DSS group), followed by the administration of 2% DSS water. The body weight of the mice began to decrease within a few days of DSS administration; however, the body weight of mice in the L164/DSS group was significantly higher than that in the DSS group on the fourth day of DSS administration (Fig. 2A). Diarrhea scores were significantly lower in the L164/DSS group than in the DSS group at the end of DSS administration (five days after DSS administration); however, no significant difference was observed in diarrhea symptoms two days after replacing DSS water with water (Figs. 2B, 2C).

At the end of the experiment, colons were collected from the DSS and L164/DSS groups. Colons in the L164/DSS group were significantly longer than those in the DSS group (Fig. 2D). Subsequently, H&E-stained sections of the colon were prepared to evaluate colonic inflammation. No significant difference was observed in colon inflammation scores between the DSS and L164/DSS groups (Fig. 2E). Consistent with this result, no

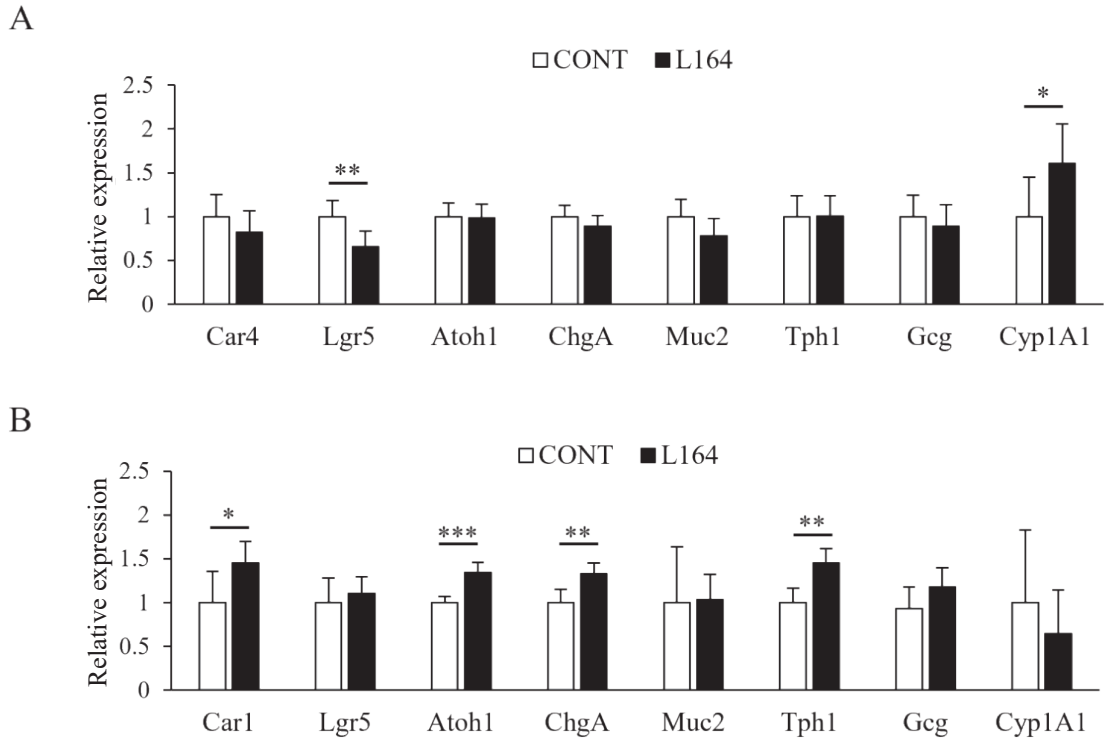

Fig. 1. Effect of L164 ingestion on stem cell markers; various differentiation markers of intestinal epithelial cells; and a sensitive marker for AhR activation in the ileum and colon
C57BL/6j mice (n = 6) were fed either a control (CONT group) or an L164-supplemented diet (L164 group). After nine days, the ileum and colon were collected from the mice. The expressions of Car4 (ileum only), Car1 (colon only), Lgr5, Aoth1, ChgA, Muc2, Tph1, Gcg, and Cyp1A1 in the (A) ileum and (B) colon were analyzed by real-time reverse transcription (RT)-PCR and normalized to the expression of β-actin. The gene expression is relative to that in the CONT group. Data represent the mean ± SD. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 (Student's t-test).

significant differences were observed in the gene expression of the inflammatory cytokines IL-1β, IL-6, and TNF-α, and the anti-inflammatory cytokine IL-10 in the colon (Fig. 3). Additionally, no significant differences were observed in the expressions of IL-22, occludin, and ZO-1 between the DSS and L164/DSS groups (Fig. 3).

Discussion

When using probiotics, live bacteria must reach the gastrointestinal tract. The L164 isolate in this study was resistant to low pH and bile treatment, comparable with LGG, suggesting its potential as a probiotic. Among the antibiotics tested in this study, L164 showed resistance only to TMP-SMX. Based on the genomic information of L164, we predicted that genes related to the resistance to TMP-SMX were present in the genome (data not presented), suggesting that L164 is unlikely to transmit its resistance to TMP-SMX to other bacteria. Therefore, L164 could be used on pig farms as a probiotic with little possibility of the transmission of antibiotic resistance.

Tph1 is the rate-limiting enzyme in the synthesis of serotonin in enterochromaffin cells (Mawe & Hoffman 2013). Serotonin released from enterochromaffin cells mediates several gastrointestinal functions, including peristalsis, secretion, and vasodilation (Mawe & Hoffman 2013). In our study, we found that L164 ingestion increased Tph1 expression in the mouse colon, suggesting that L164 may exhibit serotonin-mediated intestinal regulation. Although the mechanism through which L164 enhances Tph1 expression in the mouse colon is unknown, enterochromaffin cells are known to be subject to microbial regulation (Arora et al. 2021). Yano et al. (2015) reported that elevating concentrations of particular microbial metabolites increases colonic and blood serotonin in germ-free mice. Additionally, Liu et al. (2023) reported that supernatants of the *L. reuteri* culture significantly enhanced Tph1 expression in IPEC-J2, a porcine small intestinal epithelial cell line. Notably, future studies can be conducted to investigate the mechanism through which L164 ingestion induces Tph1 gene expression in mouse colon.

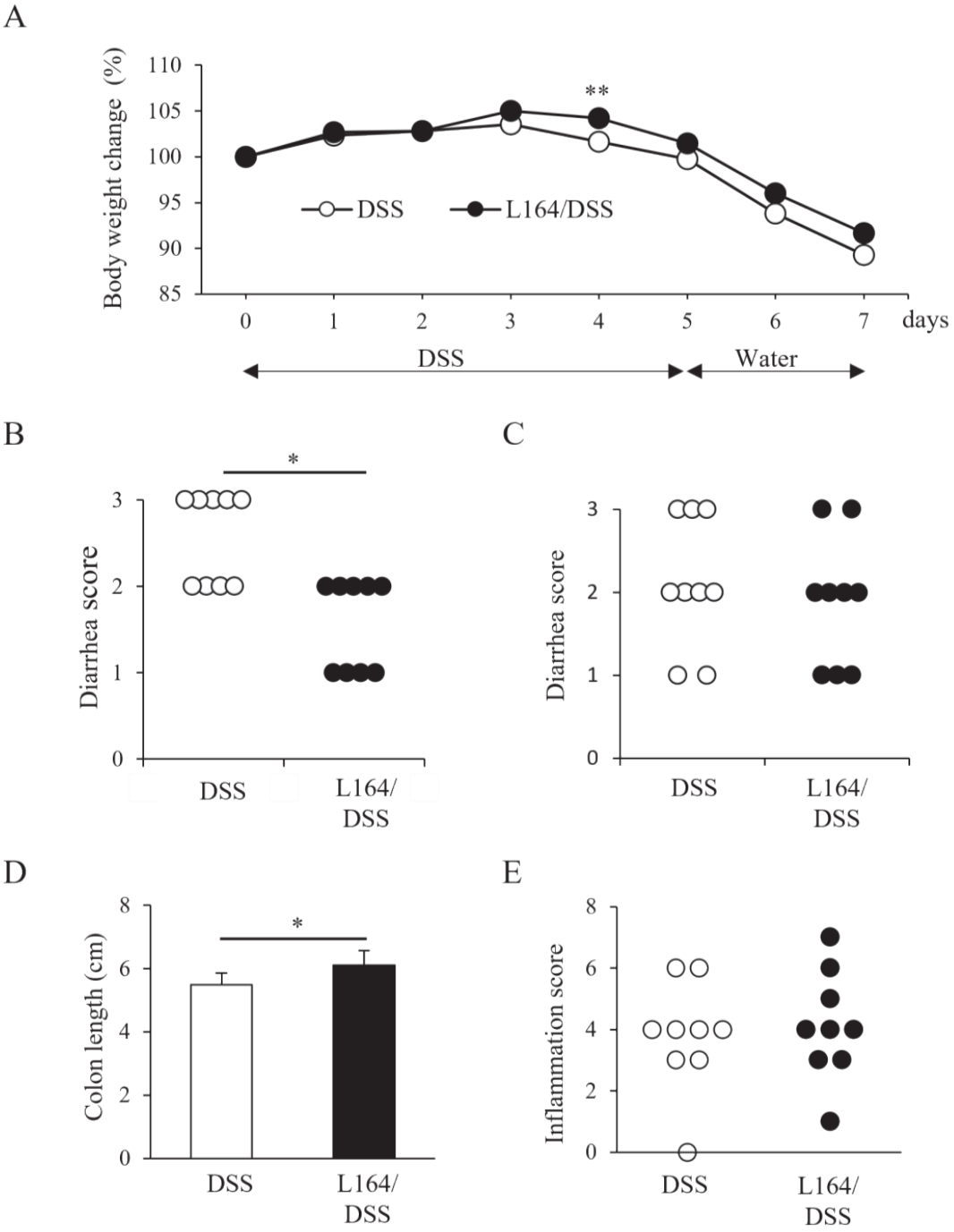

Fig. 2. Effect of L164 ingestion on DSS-induced colitis model mice

(A–E) C57BL/6j mice (n = 9 per group) were fed either a control (DSS group) or an L164-supplemented diet (L164/DSS group). After one week, the mice were allowed to drink 2% (w/v) DSS water for five days to induce diarrhea. The DSS water was then replaced with normal water for two days. (A) Body weight changes are indicated as the mean ± SD. $^{**}P < 0.01$ versus CONT group by Student's t-test. (B and C) Diarrhea scores were evaluated on the (B) fifth and (C) seventh days after the start of DSS administration. Each symbol represents the mouse. $^{*}P < 0.05$ (Mann-Whitney U test). (D) The colon length of mice in each group at the end of the experiment is indicated as the mean ± SD. $^{*}P < 0.05$ (Student's t-test). (E) Histological scores of colon sections. Each symbol represents the mouse.

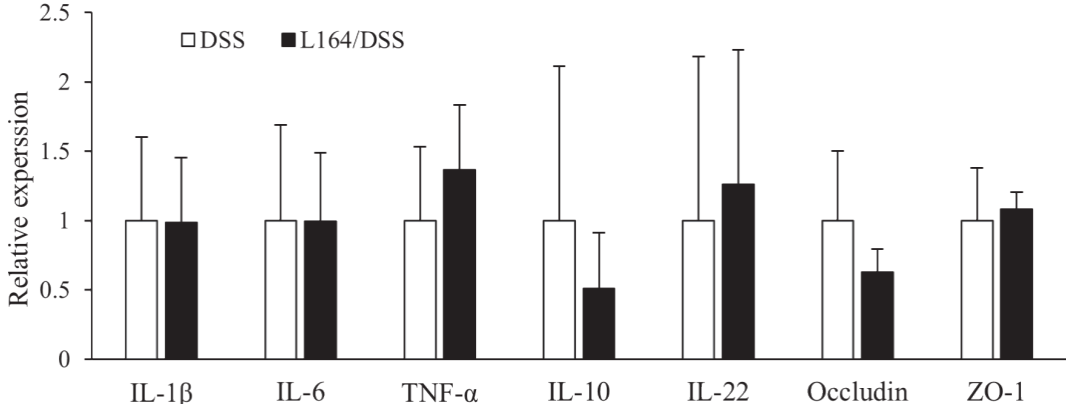

Fig. 3. Effect of L164 ingestion on gene expression in the colon of DSS-induced colitis model mice
The expressions of IL-1β, IL-6, TNF-α, IL-10, IL-22, occludin, and ZO-1 in the colon of the DSS and L164/DSS groups were analyzed by real-time RT-PCR and normalized to the expression of β-actin. The gene expression is relative to that in the DSS group. Data represent the mean ± SD.

The results of gene expression analysis of L164-administered mice indicated that L164 ingestion may activate AhR and decrease the number of intestinal epithelial stem cells in the ileum. As AhR is known to regulate intestinal epithelial stem cells (Han et al. 2020), L164 ingestion may regulate intestinal epithelial stem cells through the activation of AhR in the ileum. AhR plays an important beneficial role in the intestinal microenvironment (Stockinger et al. 2021). Therefore, L164, which has the potential to activate AhR in the ileum, may be useful for weaning piglets.

The L164 strain examined in this study showed protective effects against DSS-induced colitis. Although the DSS-induced colitis model is not a model for diarrhea in weaned piglets, L164 may be able to prevent diarrhea in weaned piglets depending on its protective mechanism of action. However, the ingestion of L164 did not induce IL-22 expression, suppress the inflammatory response, or enhance the gene expression of tight junction-related proteins, which have been reported as mechanisms of *L. reuteri*. Considering that no differences were observed in the diarrheal symptoms or colon inflammation at the end of the experiment, L164 may have suppressed the early stages of DSS-induced damage. Further studies are required to elucidate the mechanisms underlying the protective effects of L164 against DSS.

In summary, we focused on *L. reuteri* as a probiotic for weaning piglets and evaluated the extraction of L164 from pig feces as a probiotic strain. In-vitro studies have found that L164 has a tolerance to low pH and bile treatment, comparable with LGG, and that L164 is unlikely to cause problems related to antibiotic resistance. Studies on L164-administered mice have indicated that L164 ingestion may activate AhR in the ileum and increase the number of enterochromaffin cells in the colon. Furthermore, experiments using the DSS-induced colitis model showed that L164 inhibited the shortening of the colon and reduced diarrheal symptoms. Although further experiments on L164 ingestion by piglets are required, L164 may be a promising probiotic for weaning piglets.

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