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Full Paper

Protective effect of *Bifidobacterium longum* BB536 against nausea caused by pirfenidone in a mouse model of pellagra

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Pellagra is caused by abnormal intake and/or use of nicotinic acid and is known in part to be induced by the use of medications such as isoniazid or pirfenidone. We previously investigated atypical phenotypes of pellagra, such as nausea, using a mouse model of pellagra and found that gut microbiota play an important role in the development of these phenotypes. Here, we investigated the effect of *Bifidobacterium longum* BB536 on pellagra-related nausea caused by pirfenidone in our mouse model. Our pharmacological data indicated that pirfenidone (PFD) causes modulation of the gut microbiota profile, which appeared to play an important role in the development of pellagra-related nausea. A gut microbiota-mediated protective effect of *B. longum* BB536 against nausea caused by PFD was also identified. Finally, the urinary ratio of nicotinamide/N-methylnicotinamide was shown to be a biomarker of pellagra-like adverse effects induced by PFD, and it may contribute to the prevention of these effects in patients with idiopathic pulmonary fibrosis.

Key words: animal model, *Bifidobacterium longum* BB536, microbiota, pellagra, pirfenidone

INTRODUCTION

Defects in certain amino acids, such as tryptophan, affect the immune system and gut microbiota profile [1] and can cause pellagra, which is a clinical syndrome characterised by dermatitis, diarrhoea, dementia, and nausea [2–5]. The specific role of tryptophan in gut microbiota functioning is not fully understood. However, in a previous study to validate a mouse model of pellagra, we showed that deficiencies in metabolites of tryptophan, such as niacin, can cause significant changes in the gut bacterial profile [6]. Although the molecular mechanisms of pellagra have not been fully elucidated, the gut microbiota clearly plays a direct role in the development of pellagra-related symptoms [6].

Pellagra has been reported to be divided into two types [5]. One type may result from insufficient intake of dietary niacin or tryptophan (primary pellagra), and the other results from insufficient use of niacin or tryptophan (secondary pellagra). Isoniazid causes secondary pellagra in humans and rodents, and its phenotype, including photosensitivity, has been well investigated [7, 8]. Recently, we found that isoniazid also causes pellagra-related nausea in a new mouse model we established [9]. Using this mouse model, some adverse effects (AEs) induced by pirfenidone (PFD) were recognised to be similar to pellagra [10]. Furthermore, strong changes in the gut microbiota of db/db mice treated with PFD were observed compared with that of untreated mice [11]. Importantly, to date, mechanistic insights into PFD-related pellagra via the gut microbiota have not been fully elucidated. Further, the direct involvement of gut microbiota has also not been investigated.

Prevention of adverse drug reactions in patients with idiopathic pulmonary fibrosis (IPF) is critical to maintain their quality of life, as many of these patients will have their PFD doses reduced or even discontinue taking PFD because of AEs [12]. An interesting approach to attenuating AEs of PFD is the use of a respirable powder formulation to reduce exposure [13]. Therefore, we investigated another methodology for reducing AEs thought to be pellagra-related symptoms, one that involves controlling the gut microbiota. A prebiotic and probiotic supplement (Kenchou Keikaku Totonoale, Shionogi Healthcare Co., & Ltd., Osaka, Japan) containing *Bifidobacterium longum* BB536 (BB536) was used in this study because some studies have indicated that BB536 has some beneficial and positive effects on human health via gut microbiota-modulating properties [14, 15].

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Here, we investigated the therapeutic effect of the probiotic supplement on pellagra development caused by PFD using the appropriate mouse model. Although further verification is needed, AEs in IPF patients receiving PFD may be alleviated by controlling the gut microbiota.

MATERIALS AND METHODS

Experimental animals

Six-week-old female Balb/c mice (Japan SLC, Hamamatsu, Japan) were raised in groups of 5 mice per cage as described previously [6, 9] and fed normal (AIN-93G) and low-niacin (modified AIN-93G) diets (Oriental BioService, Kyoto, Japan) with specific vitamin compositions [6]. Mice were housed under controlled environmental conditions (24 ± 2°C; 50% ± 20% relative humidity; 12 hr light/dark cycle, lights on at 8:00 a.m.) and weighed every 2 days. A total of 120 mice were used in the present study.

The study was approved by the Institutional Animal Care and Use Committee of Shionogi & Co., Ltd. Procedures were conducted in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (S21041C-0000).

Experimental design

In experiment 1, mice were allocated to six groups (n=4 per group). After 10 days of feeding on the normal or low-niacin diet, mice were treated with the vehicle (saline) or PFD (1 or 3 mg/mouse; Shionogi Co., Ltd., Osaka, Japan) twice a day for 6 days. At 5 days after the first treatment, faeces and urine were harvested and stored at −80°C until analysis.

In experiment 2, mice were allocated to four groups (n=4–9 per group). After 10 days of feeding on the low-niacin diet, mice were treated with the vehicle or PFD (3 mg/mouse) twice a day for 6 days. Some mice were treated with BB536 using HydroGel (ClearH_2O, Inc., Westbrook, ME, USA), a non-wetting water gel for animal hydration. Briefly, to melt the gel, cups of gel were incubated at 60°C in a deep-well maximiser (TAITEC, Tokyo, Japan) with shaking at 120 rpm. After melting, 1.5 g of Kenchou Keikaku Totonoale (2×10^9 colony forming units (CFU) of BB536) was added to the cup, which was then shaken vigorously and stored at 4°C until use (within 4 hr of preparation).

Just before the first administration of PFD, one cup of gel with or without BB536 was placed in the cage with the mouse. Cups were changed twice daily to minimize contamination. When the gel was introduced, the use of water nozzles was discontinued. At 5 days after the first treatment, faeces and urine were harvested and stored at −80°C until analysis.

Analysis of gut microbiota

Analysis of the gut microbiota was carried out as described previously [6]. Briefly, faeces were collected in 2 mL tubes, subjected to freeze-drying, and crushed using a Multi-beads Shocker. DNA was extracted and purified from the crushed faeces using ISOSPIN Fecal DNA (Nippon Gene, Tokyo, Japan) and MPure Bacterial DNA Extraction (MP Bio Japan, Tokyo, Japan) kits in accordance with the manufacturers' procedures. A DNA library was constructed using a two-step tailed polymerase chain reaction method. The quality of the constructed libraries was checked, and each read sequence was extracted for which the beginning of the sequence exactly matched the primer used. Using the extracted sequences without the primer sequence, read sequences with the appropriate quality were selected and joined with others that had at least a 10 bp overlap.

The delta score was calculated to estimate differences in the bacterial profiles among groups in accordance with previous reports [6, 16].

Analysis of niacin and its metabolites

The urine concentrations of niacin and its metabolites were analysed as described previously [9]. Briefly, mouse urine samples were diluted 20-fold with Milli-Q water, and a 10 µL aliquot of urine was combined with 70 µL of an internal standard solution comprised of methanol spiked with a deuterium-labelled internal standard. The deuterium-labelled internal standard contained nicotinamide (NAM)-d4, N-methyl-2-pyridone-5-carboxamide (2-Py)-d3, N-methyl-4-pyridone-5-carboxamide (4-Py)-d3, methylnicotinamide (MNA)-d3, creatinine-d3, nicotinamide-N-oxide (NNO)-d3, nicotinic acid (NA)-d4, tryptophan-d5, kynurenic acid-d5, kynurenine-d4, and xanthurenic acid-d4. The samples were diluted 5-fold with Milli-Q water. Finally, 3 µL of each sample was injected into a liquid chromatography (LC)/mass spectrometry (MS)-MS system. The separation conditions for LC/MS were based on a previous study we performed [9].

Quantitative analysis of pellagra-related pica

In accordance with the aforementioned previous study [9], nausea was quantified by quantifying the red staining caused by pica behaviours in images of mouse faeces using the ImageJ software. Briefly, mice were provided with paper tips stained with carminic acid (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan), and they fed on these paper tips as a result of nauseous behaviour. Next, stained faeces were recovered, and images with a constant focal length were obtained using a digital camera (D100; Nikon, Tokyo, Japan), divided based on three colours (red, blue, and green), and converted to greyscale. Using ImageJ, blue staining was subtracted from red staining, and the remaining colour was then quantified.

Statistical analysis

The sample size was determined to be n=4–9 per group in accordance with a previous study [6]. Data were expressed as the mean ± standard error of the mean. GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA) was used for the statistical analysis. The paired t-test, Wilcoxon rank-sum test, or Tukey's multiple comparisons method was used.

RESULTS

Effect of PFD on the gut microbiota

We previously reported that PFD caused pellagra-related nausea in mice fed a low-niacin diet, but not in those fed a normal diet [17]. Furthermore, the gut microbiota plays a major role in the development of pellagra-related nausea in mice [6]. In accordance with these findings, we investigated the effect of PFD on gut microbiota. The study design is summarised in Fig. 1A. As shown in Fig. 1B, kinetic changes in body weight did not differ among the groups. Next, the gut microbiota was analysed in faeces harvested from the mice beginning 15 days after initiating the experiments. As shown in Fig. 2A, some unique orders, such

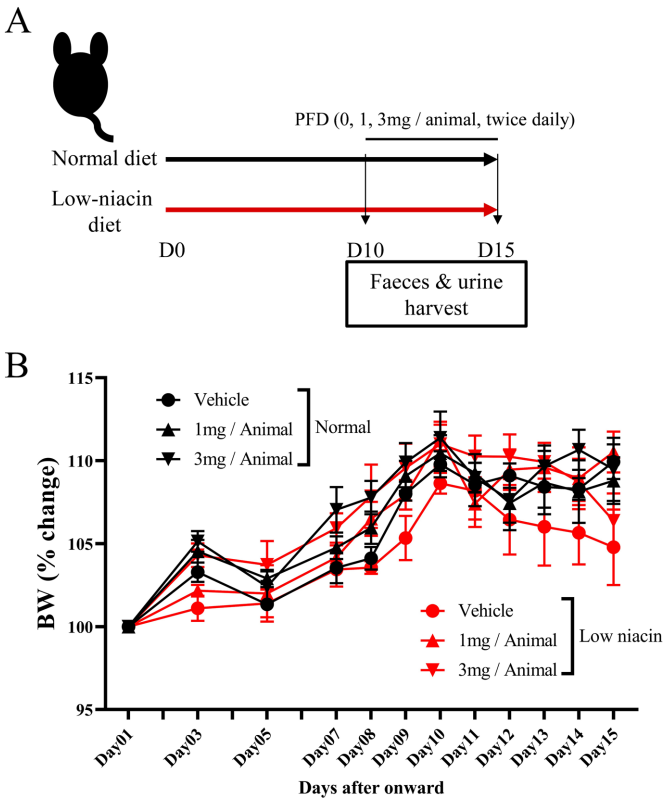

Fig. 1. Procedures of animal experiments to investigate the role of PFD in the gut microbiota. (A) The black arrow indicates mice on the normal diet. The red arrow indicates mice on the low-niacin diet. The experiments started on day (D) 0. Vehicle or PFD (twice daily for 5 days) was given from D10 to D15. Urine was collected on D10, D11, D14, and D15. Faeces were collected on D15. (B) Growth curves (% change in body weight) in the normal and low-niacin diet groups (n=5 in each group). Each comparison was carried out among groups. ●, vehicle; ▲, PFD (1 mg/animal); ▼, PFD (3 mg/animal); Black, normal diet; red, low-niacin diet. PFD: pirfenidone; BW: body weight.

as *Bacteroidales*, were observed in mice fed the normal diet and treated with PFD compared with those fed the normal diet but not treated with PFD. A higher *Clostridiales* response to PFD (3 mg/animal) was also observed compared with PFD (1 mg/animal)- or vehicle-treated mice fed the normal diet. Interestingly, diet had a significant effect on the gut microbiota profile. When we focused on individual bacteria, statistically lower *Streptococcaceae* responses were observed in mice fed the normal diet and treated with PFD (1 or 3 mg/animal) compared with those treated with the vehicle and were also observed in mice fed the low-niacin diet and treated with the vehicle compared with those fed the normal diet (Fig. 2B). A low response to PFD (p=0.08) was observed in mice fed the low-niacin diet and treated with PFD (3 mg/animal) compared with those treated with the vehicle. As shown in Fig. 1C, delta scores were determined in accordance with previous studies [6, 16] to estimate differences in the bacterial profiles among groups. The score was significantly higher in mice fed the normal or low-niacin diet and treated with PFD compared with those treated with the vehicle.

Effect of PFD on pellagra-related nausea

The urinary ratio of NAM/MNA in mice treated with or without PFD was investigated to evaluate whether nausea caused by PFD was similar to that caused by pellagra. This is because abnormal niacin metabolism occurs in pellagra [17, 18], and this urinary ratio may represent a suitable biomarker for pellagra [6, 19]. As shown in Fig. 3A, there were no significant differences in the ratio among the groups before treatment (day 10). A higher ratio was observed in mice fed the normal or low-niacin diet and treated with PFD compared with those treated with the vehicle at 11 days after beginning the experiment (immediately after PFD treatment). A higher ratio was also observed in mice fed the normal diet, but not the low-niacin diet, and treated with PFD compared with those treated with the vehicle at 14 and 15 days after beginning the experiment. The clinical features of faeces from mice fed the normal or low-niacin diet and treated with PFD or the vehicle are shown in Fig. 3B. Faeces stained red indicated nauseous behaviour in mice fed the low-niacin diet and treated with PFD. The nausea score was significantly higher in mice fed the low-niacin diet and treated with PFD compared with those treated with the vehicle or fed the normal diet (Fig. 3C).

Effect of BB536 on pellagra-related nausea mediated via the gut microbiota

BB536 plays a role in maintaining human health by modulating the gut microbiota [14, 20, 21]. Furthermore, loss of gut bacteria caused by antibacterial agents causes pellagra-related nausea in mice [6]. Therefore, we investigated the effect of BB536 on the development of pellagra-related nausea by modulating the gut microbiota in accordance with the procedure shown in Fig. 4A. As shown in Fig. 4B, weight gain stagnated temporarily in mice fed the low-niacin diet and treated with PFD at 12 and 13 days after beginning the experiment. Weight gain was confirmed by the time pica behaviour started in these mice. As shown in Fig. 5A, prominent effects of BB536 were exerted on the gut microbiota profile. For example, a lower *Erysipelotrichales* response was observed in mice treated with BB536 compared to those that were not. Interestingly, a higher *Lactobacillales* response to BB536 was also observed in the group treated with both PFD and BB536 compared with the group treated with PFD alone. As shown in Fig. 5B–5D, individual bacterial responses to PFD and/or BB536 were observed. Although a *Lactobacillaceae* response was not found in mice treated with BB536, a significantly higher response to BB536 was observed in mice treated with PFD compared with untreated mice. A higher *Streptococcaceae* response to BB536 was observed in mice treated with or without PFD. *Erysipelotrichaceae* responses to BB536 were significantly higher in mice treated with the vehicle or PFD. Furthermore, a higher *Rikenellaceae* response was observed in mice treated with PFD compared with mice treated with the vehicle, and this was negated by BB536. As shown in Fig. 5E, the delta score of the bacterial profile using faeces from each mouse group was investigated to evaluate overall responses rather than the responses of individual bacterial families. Scores were significantly higher in BB536-treated mice and were similar between vehicle- and PFD-treated groups.

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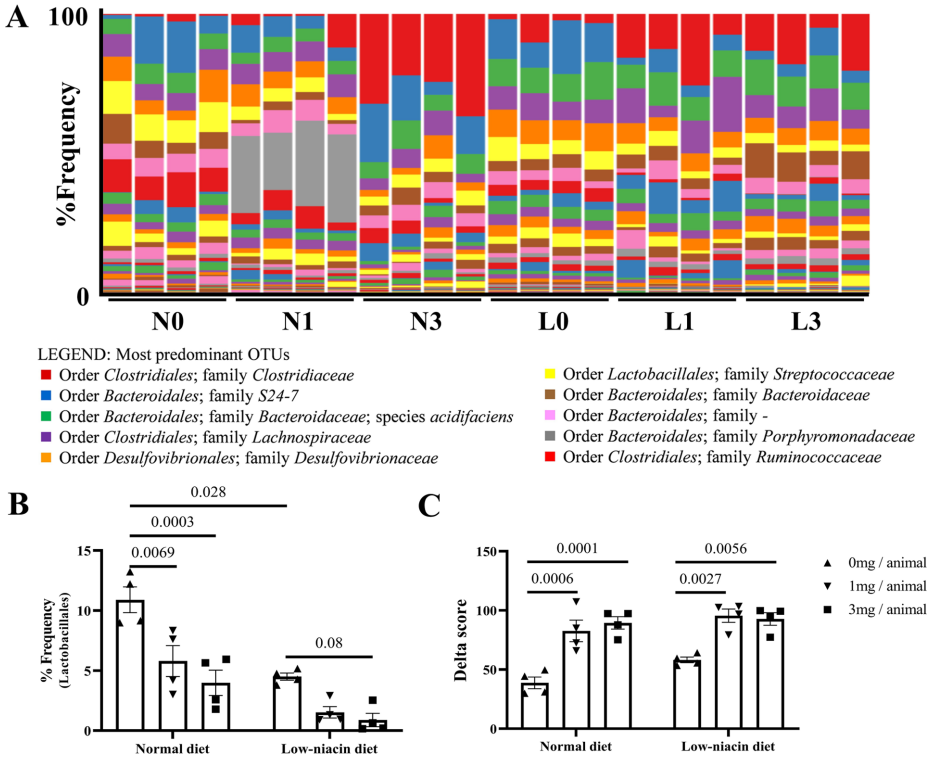

Fig. 2. Effect of PFD on the gut microbiota. (A) Operational taxonomic units (OTUs) of gut microbes were compared between mice treated with the vehicle or PFD and fed the normal diet (n=4) or low-niacin diet (n=4 in each group). N0, normal diet with vehicle; N1, normal diet with PFD (1 mg/animal); N3, normal diet with PFD (3 mg/animal); L0, low-niacin diet with vehicle; L1, low-niacin diet with PFD (1 mg/animal); L3, low-niacin diet with PFD (3 mg/animal). The most predominant OTUs are indicated. (B) *Lactobacillales* responses to each treatment are indicated. (C) Delta scores were calculated to estimate differences in bacterial profile among groups in accordance with a previous study [6]. The experiments were repeated twice. Each symbol represents an individual. ▲, vehicle; ▼, PFD (1 mg/animal); ■, PFD (3 mg/animal). Data represent the mean ± standard error. Statistical analysis was conducted as stated in the Materials and Methods.

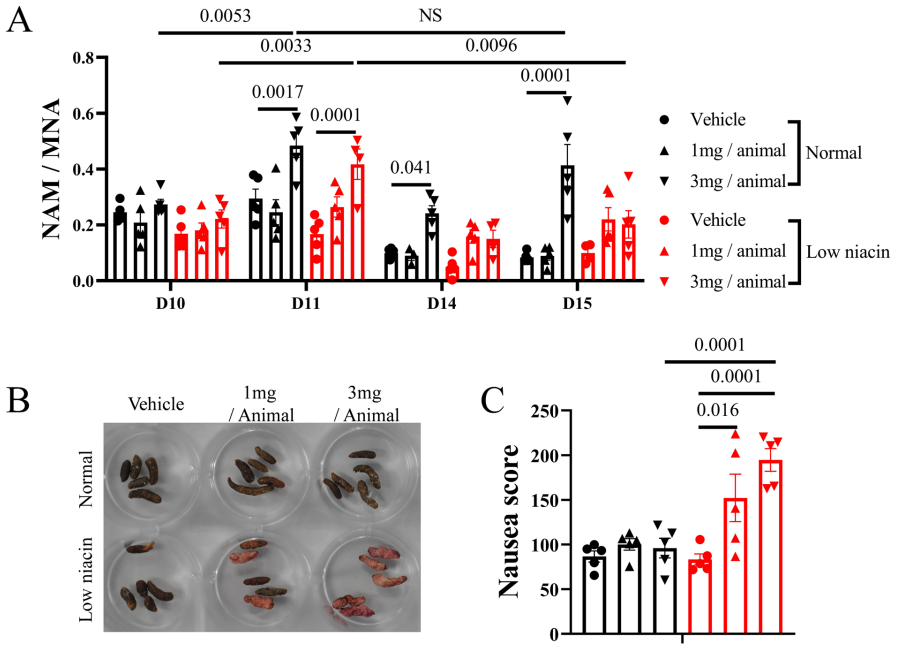

Fig. 3. Pellagra-related nausea caused by PFD. (A) The NAM/MNA ratio was determined to evaluate whether pellagra developed in the mice or not (n=4–5 in each group). (B) Faeces were collected from mice given paper tips stained with carminic acid; coloured faeces indicated nausea. (C) The degree of nausea was scored using colour as an index. The experiments were repeated twice. Each comparison was carried out among groups. ●, vehicle; ▲, PFD (1 mg/animal); ▼, PFD (3 mg/animal); Black, normal diet; red, low-niacin diet. Data represent the mean ± standard error. Statistical analysis was conducted as stated in the Materials and Methods. PFD: pirfenidone; NAM: nicotinamide; MNA: methylnicotinamide; NS: not significant.

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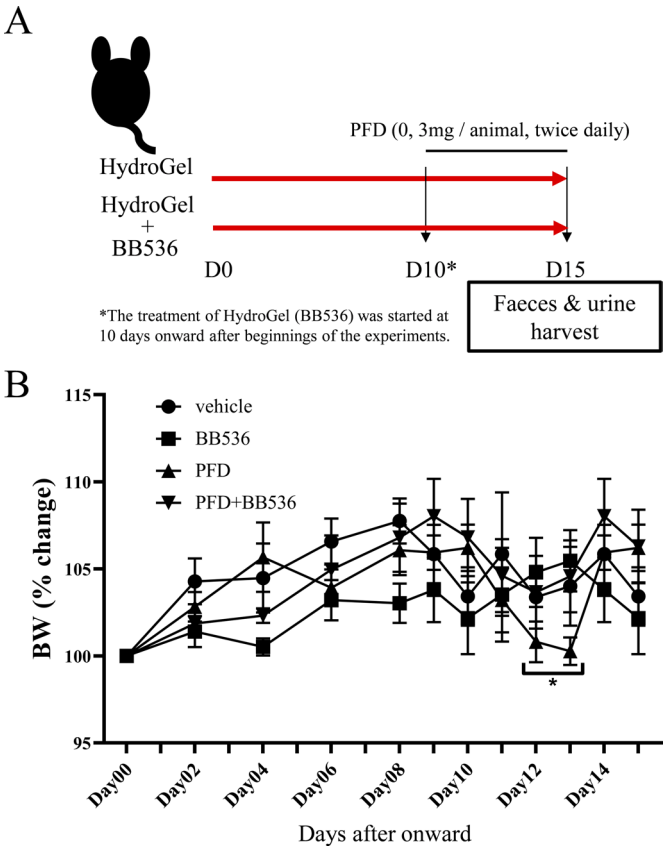

Fig. 4. Procedures of animal experiments to investigate the role of BB536 in the development of pellagra-related nausea. (A) A red arrow indicates mice on the low-niacin diet. The experiments started on day (D) 0. The upper arrow indicates mice that were not treated with BB536, while the lower indicates mice treated with BB536. The vehicle or PFD (twice daily for 5 days) was administered from D10 to D15. Urine and faeces were collected on D15. (B) Growth curves (% change in body weight) in mice treated with the vehicle, BB536, PFD, or BB536 + PFD and fed the low-niacin diet (n=5–9 in each group). Each comparison was carried out among groups. ●, vehicle; ■, BB536; ▲, PFD; ▼, PFD + BB536. PFD: pirfenidone; BW: body weight. *Temporary stagnation in weight gain.

Beneficial effect of BB536 on pellagra-related nausea

Kinetic changes in the NAM/MNA ratio appear to represent an appropriate biomarker to evaluate pellagra-like symptoms, as shown by the results shown in Fig. 3A. When the ratio increased transiently and then decreased, mice had developed pellagra. However, mice did not develop pellagra when the ratio remained high. As shown in Fig. 6A, a slightly higher response to BB536 was observed in mice treated with PFD at 15 days after the beginning of the experiment compared with those treated without BB536. Pica behaviour, which is an indicator of pellagra-related nausea in mice [9], was observed in mice treated with PFD (Fig. 6B). Next, nausea was evaluated quantitatively using carminic acid-stained paper tips that were eaten by mice with pica. As shown in Fig. 6C, mice treated with PFD had a significantly higher nausea score compared with those treated with the vehicle, and this was negated by BB536.

DISCUSSION

Nutritional status plays a major role in disease prognosis, and several factors responsible for disease progression have a negative effect on malnutritional status, although many IPF patients are not underweight at the time of diagnosis and may even be overweight [22, 23]. Some studies have discussed this disease by referring to specific nutrients, such as saturated fatty acids, polyunsaturated fatty acids, amino acids, and vitamins [24]. However, no reports have investigated the AEs of PFD from a nutritional viewpoint. Recently, we reported that niacin status is the major factor influencing AEs caused by PFD [10]. To date, however, the benefits of niacin derived from gut bacteria have not been reported. In this study, we found that a nutritional approach using *B. longum*, which produces high levels of niacin [25] and is widely used as a nutritional supplement, had a beneficial effect on the development of pellagra-related symptoms by modulating the gut microbiota and that kinetic changes in the NAM/MNA ratio may be a biomarker for evaluating pellagra-related nausea caused by PFD. Importantly, PFD is widely used to treat IPF patients, some of whom suffer from side effects [12]. Changes in the gut microbiota profile caused by a low-niacin diet are transient [6], and the intestinal environment was readily modulated using BB536 in the present study (Fig. 5). Furthermore, a previous study using germ-free mice indicated that gut bacteria play an important role in preventing nausea by maintaining the niacin status in mice fed a low-niacin diet [6]. Therefore, the gut microbiota might represent a therapeutic target for PFD-related pellagra.

Although the composition of the gut microbiota can vary significantly between individuals, it appears to be relatively stable within individuals but to become more diverse and variable with age [26, 27]. Various biomolecules, nutrient signalling-independent pathways, and epigenetic mechanisms from the host affect the gut microbiota profile. Defects in such communication between the host and bacteria caused by age-related gut dysfunction can affect host health and longevity [28]. Some reports have indicated that BB536 has a beneficial effect on human health by modulating the gut microbiota [14, 15, 20]. Interestingly, a synergic effect of PFD and BB536 on *Lactobacillaceae,* which are food-fermenting lactic acid bacteria [29], was observed in the present study, as shown in Fig. 5B. A significantly low response of *Erysipelotrichaceae* to PFD, which may be a potential inflammatory marker in HIV patients [30], was also observed.

A significantly high response to PFD by *Rikenellaceae*, which may be a potential biomarker for selecting patients with type 2 diabetes mellitus to receive faecal microbiota transplantation [31], was negated by BB536. These unique profiles caused by BB536 might contribute to a protective effect of PFD against pellagra-related nausea. Even in a comprehensive analysis using the delta score rather than focusing on individual bacterial species, BB536 appeared to have a strong effect on the gut microbiota compared with PFD.

The gut microbiota, which is strongly influenced by the host, is sometimes altered transiently during viral respiratory infections [32]. These transient changes are also caused by a low-niacin diet [6]. Species such as S24-7, *Desulfovibrionaceae, Bacteroidaceae (acidifacines), Lachnospiraceae,* and *Porphyromonadaceae* were dominant in faeces from mice fed both the vehicle and the

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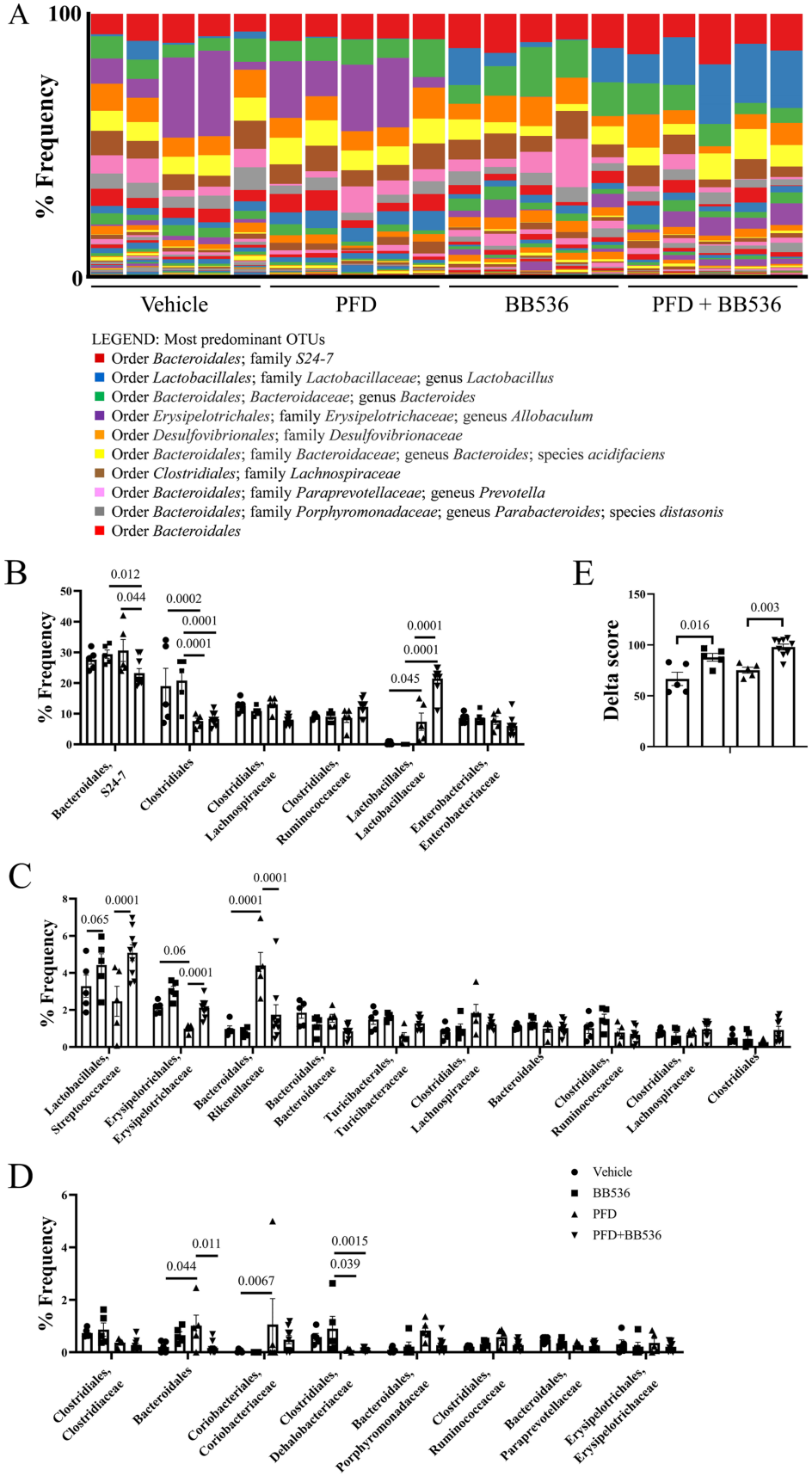

Fig. 5. Effect of BB536 on the gut microbiota. (A) Operational taxonomic units (OTUs) of gut microbes were compared between mice treated with the vehicle, PFD, BB536, or PFD + BB536 and fed the low-niacin diet (n=5). (B–D) Bacteria responses to each treatment are shown. (E) Delta scores were calculated to estimate differences in the bacterial profile among groups, as described previously [6]. The experiments were repeated twice. Each symbol represents an individual. Each comparison was carried out among groups. ●, vehicle; ■, BB536; ▲, PFD; ▼, PFD + BB536. Data represent the mean ± standard error. Statistical analysis was conducted as stated in the Materials and Methods. PFD: pirfenidone.

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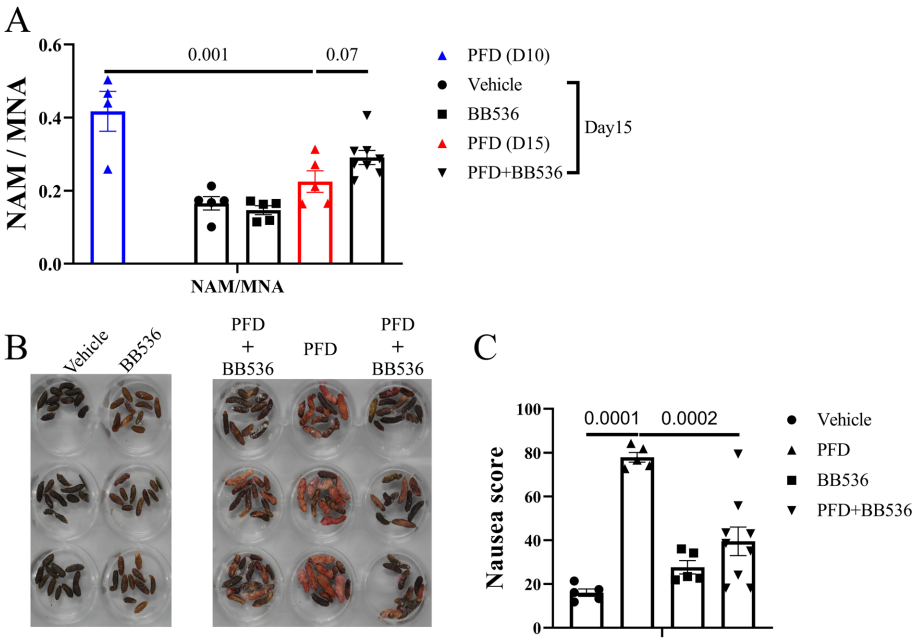

Fig. 6. Effect of BB536 on nausea development induced by PFD. (A) The NAM/MNA ratio was determined to evaluate whether or not pellagra developed in these mice (n=5–9 in each group). (B) Faeces were collected from mice given paper tips stained with carminic acid; coloured faeces indicated nausea. (C) The degree of nausea was scored using colour as an index. Each symbol represents an individual. Each comparison was carried out among groups. ●, vehicle; ■, BB536; ▲, PFD; ▼, PFD + BB536. Data represent the mean ± standard error. Statistical analysis was conducted as stated in the Materials and Methods. PFD: pirfenidone; NAM: nicotinamide; MNA: methylnicotinamide.

low-niacin diet. Between the two experiments (Figs. 2 and 5), *Erysipelotrichaceae* has a large difference in its abundance. This family was dominant in mice kept under low-niacin diet with vehicle (Fig. 5) but not mice kept under low-niacin diet with vehicle (Fig. 2). The *family* ranked 11th, the next dominant species after *Ruminococcaceae* in Fig. 2. We therefore concluded that the bacterial profile was similar following treatment with PFD and BB536, although some discrepancies were observed. For example, the order of dominant families differed between the treatment groups and may have been attributable to differences in the mechanism of water supply (an automatic water supply device versus a water supply gel that was replaced twice daily to minimize contamination).

Nausea was evaluated quantitatively using mice with pica behaviours in accordance with a previous study [9]. We used organic solvents to extract red coloration from faeces and digital imaging to extract coloration without the use of solvents, and we have previously reported that results using these methods correlate statistically [9]. It is important to use images with the same focal length for digital procedures in the same experiment because the use of different focal lengths for images of the same faeces results in different nausea scores, as shown in Figs. 3 and 4.

It is also important to establish a method to avoid side effects and exert the maximal therapeutic effect because some IPF patients will have their PFD doses reduced or even discontinue treatment because of AEs [12]. To date, several methods to avoid side effects have been proposed, but none have been put into clinical use [13]. Here, we demonstrated the utility of BB536 in a mouse pellagra model established by PFD from the viewpoint of reducing AEs. We thought that whether or not the beneficial properties presented here were unique to BB536 was of scientific interest. On the other hand, it is not considered characteristic of BB536, as many bacteria exhibit niacin responsiveness [33]. In order to investigate these issues, we thought that it would be necessary to prepare BB536 that does not produce niacin, but this is not realistic or would be too difficult for us. More importantly, further research is needed prior to clinical use of BB536 because its safety has not yet been evaluated in IPF patients.

CONFLICT OF INTEREST

Tomohiro Kuroita, Natsumi Susai, and Takeshi Yoshioka are employees of Shionogi & Co., Ltd. Other authors declare no competing interests.

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