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*Research article*

# Preventive effect of *Enterococcus faecalis* and β-glucan on the onset and exacerbation of symptoms of atopic dermatitis in mice

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**Abstract:** Atopic dermatitis (AD) is associated with an immune response caused by the excessive production of IgE antibodies. This is accompanied by excessive itching as a clinical symptom, leading to a great burden and anxiety in daily life. At present, there is no established treatment that can completely cure AD, and symptomatic treatment alleviates symptoms to the extent that they do not interfere with daily life. Recent research has suggested that the ingestion of either various lactic acid bacteria or β-glucan suppressed the production of IgE antibodies and alleviated the symptoms of AD. Therefore, in this study, we used a drug-induced AD mouse model to evaluate the amelioration effect of EC-12 and β-glucan derived from black yeast to investigate the mechanism involved. The oral administration of lactic acid bacteria inhibited the expression of inflammatory cytokine genes at the lesion site, and the administration of β-glucan downregulated serum IgE levels and inhibited the expression of inflammatory cytokine genes at the lesion site. The concomitant administration of EC-12 and β-glucan strongly suppressed AD symptoms, downregulated serum IgE levels, and inhibited the expression of inflammatory cytokines at the lesion site. In summary, the oral administration of EC-12 and β-glucan prevented AD in mice.

**Keywords:** synbiotics; β-glucan; *Enterococcus faecalis*; aureobasidium pullulans; atopic dermatitis

**Abbreviations:** AD: atopic dermatitis; DC: dendritic cells; DNFB: 2,4-dinitrofluorobenzene; ELISA: enzyme-linked immuno-sorbent assay; GR: glucocorticoid receptor

## 1. Introduction

Atopic dermatitis (AD) is a chronic eczematous lesion accompanied by excessive itching [1]. Many people, mainly in developed countries including Japan, suffer from AD and the incidence rate is estimated to be about 30% in children and about 10% in adults [2]. AD can occur anywhere on the body, though it usually starts in exposed areas such as the face and arms, which places a heavy mental and physical burden on patients, potentially leading to a decline in their quality of life [3]. Recent studies on the onset mechanism of AD reported that several factors might be involved including skin barrier dysfunction related to excessive scratching behavior, stimuli from external environmental factors [4], genetic predisposition, and immune responses [5]. Treatment of AD is based on the pathology, typically consisting of topical steroids or ointments to moisturize the skin and suppress inflammatory reactions against the physiological dysfunction of the skin. Unfortunately, antihistamines and antiallergic drugs are not very effective and pruritus may not be sufficiently controlled [6].

Many studies have reported that lactic acid bacteria (LAB) can improve the allergy symptoms and either delay or prevent its onset [7]. The mechanism of their antiallergic action has been mainly investigated by oral administration tests using mice and haptens, which are low-molecular compounds that induce an immune response [8]. A hapten becomes an antigen by binding to macromolecules such as proteins. When a representative hapten such as 2,4-dinitrofluorobenzene (DNFB) is applied to the skin of mice, it binds to proteins as it penetrates the skin, is taken up by antigen-presenting cells, and is distributed to regional lymph nodes, where it activates lymphocytes [9]. AD occurs when DNFB is applied at regular intervals after the first sensitization [10]. Skin swelling, which is a termed delayed type reaction, is induced after several applications of DNFB. Immunoglobulin E (IgE) plays an important role in the delayed type reaction, and is clinically characterized within AD [11]. AD with a predominant IgE background induced by the repeated application of DNFB is thought to reproduce some of the characteristics of AD and has been used as an experimental model. In this study, we used a DNFB-induced AD mouse model that exhibits symptoms similar to those of human AD.

Recent studies have demonstrated that paraprobiotic *Enterococcus Faecalis* improves inflammation-associated colon carcinogenesis in mice [12], and that *Enterococcus faecalis* EC-12 (EC-12), which is obtained by heat-sterilizing *Enterococcus faecalis* isolated from the human intestinal tract, prevents the development of intestinal mucositis in mice [13].

The purpose of this study was to test whether EC-12 could prevent the onset of AD in mice, with the ultimate goal of developing therapeutic agents for human AD in the future.

## 2. Materials and methods

### *2.1. Bacteria and β-glucan*

EC-12 and β-glucan were gifted from Inaba-foods Co. Ltd. (Shizuoka, Japan). For oral administration, freeze-dried bacterial cells were suspended at $1 \times 10^8$ cfu/2 mg/100 μL PBS. β-glucan derived from black yeast was suspended at 165 mg/100 μL PBS.

*2.2. Mice*

Female BALB/cJ mice (5-weeks old) and standard chow were purchased from Clea Japan and housed under conventional conditions. The mice were used to assess the immunosuppressive effect of EC-12 and/or β-glucan on AD.

*2.3. Sensitization of mice with DNFB*

AD phenotypes in mice can be reproducibly generated by applying haptens, such as DFNB, on their dorsal skin. A preliminary experiment confirmed that DNFB induces inflammatory changes on their dorsal skin (Figure S1). To induce AD, we shaved the back of mice and sensitized them by painting 100 μl of 0.15% DNFB in vehicle (acetone/olive oil (3:1)) once a week for 5 weeks. For the oral administration of EC-12, 100 μL of PBS solution containing 2 mg of freeze-dried bacterial cells was administered to the stomach by a daily single oral gavage with a feeding needle. Using the same treatment regimen, mice in the control group orally received the same volume of PBS only. The induction schedule of AD in mice by the repeated application of DNFB, EC-12, and/or β-glucan is shown in Figure 1. AD mice were divided into four groups: (1) mice receiving vehicle only (control), (2) mice receiving EC-12, (3) mice receiving β-glucan, and (4) mice receiving EC-12 and β-glucan.

*2.4. Ethics approval of research*

These animal experiments were carried out in accordance with the guidelines of the Bioscience Committee of Hokkaido University and were approved by the Animal Care and Use Committee of Hokkaido University.

*2.5. Histological analysis*

The dorsal skin tissue was removed from mice receiving vehicle only and each group of mice receiving EC-12 and β-glucan on the last day, as shown in Figure 1, and fixed in 10% formalin (Wako Pure Chemical Industries, Japan). Sections were prepared and stained with hematoxylin and eosin at Morpho Technology, Japan. The sections were observed by a microscope equipped with a CCD camera (KEYENCE VB-7000).

*2.6. Enzyme-linked immuno-sorbent assay (ELISA)*

Blood samples (100 μL) were collected from the hind legs of mice using a Golden ANIMAL LANCET (Bio Research Center) and transferred into BD Microtainer Tubes (Becton, Dickson and Company). Sera from AD mice were obtained after centrifugation. Total IgE levels were measured by ELISA according to the manufacturer's instructions (BD Biosciences).

*2.7. RNA extraction and real-time quantitative PCR*

Dorsal skin tissue was removed from each group on the last day, as shown in Figure 1, and skin (100 mg) was homogenized in 1 mL TRIZOL reagent (Invitrogen). Total RNA was extracted

according to the manufacturer's instructions. Subsequently, 1 μg of RNA was reverse-transcribed to cDNA using ReverTra Ace (TOYOBO) according to the manufacturer's instructions. Using a KAPA SYBR Fast qPCR Kit (Nippon Genetics), reactions were run on a Step One Plus real-time PCR machine (Life Technologies) according to the manufacturer's instructions. Data were normalized using the ΔΔCt method and a sample from the vehicle group was used as a calibrator. Primers used for real-time PCR were as follows:

GAPDH forward 5′-AAGGGCTCATGACCACAGTC-3′,
GAPDH reverse 5′-GGATGCAGGGATGATGTTCT-3′,
TNF alpha forward 5′-AGCCCACGTCGTAGCAAACCAC-3′,
TNF alpha reverse 5′-CGGGGCAGCCTTGTCCCTTG-3′,
IL-6 forward 5′-CGTGGAAATGAGAAAAGAGTTGTGC-3′,
IL-6 reverse 5′-TGGTACTCCAGAAGACCAGAGGA-3′,
IL-10 forward 5′-GCCCCAGGCAGAGAAGCATGG-3′,
and IL-10 reverse 5′-GGGGAGAAATCGATGACAGCGCC-3′.

### *2.8. Statistical analysis*

All data are reported as the mean ± SD. Statistical analysis of the data was performed by one-way ANOVA. Statistical significances were determined by ANOVA, followed by Fisher's protected least significant difference, and significances where $P < 0.05$ and $< 0.01$ are indicated by respective symbols in the figures.

## 3. Results

### *3.1. A mixture of EC-12 and β-glucan attenuates DNFB-induced AD symptoms*

First, we examined the efficacy of the oral administration of EC-12 and/or β-glucan to attenuate DNFB-induced AD in mice. The application schedule of DNFB, EC-12, and/or β-glucan is shown in Figure 1. The oral administration of a mixture of EC-12 and β-glucan significantly suppressed back thickening observed in the control mice (Figure 2), which in turn suppressed clinical scores representative skin lesions observed in AD compared with the other experimental groups (Figure 3). In addition, histological examination showed thickening of the skin in the backs of the control AD mice, which was not present in mice administered with a mixture of EC-12 and β-glucan (Figure 4). These findings indicated the potential use of a mixture of EC-12 and β-glucan to prevent AD.

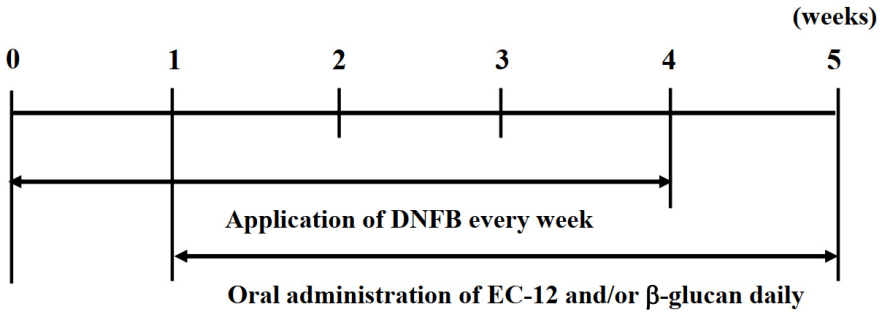


**Figure 1.** Experimental schedule. Female BALB/cJ mice (6-weeks old) were sensitized by applying 100 μl of 0.15% DNFB in vehicle (acetone/olive oil (3:1)) once a week for 5 weeks. For daily oral administration, freeze-dried bacterial cells were suspended in PBS at $1 \times 10^8$ cfu/2 mg/100 μL. β-glucan was suspended in PBS at 165mg/100 μL. These bacteria (EC-12) and/or β-glucan were administrated orally once daily.


**Figure 2.** Effects of oral administration of EC-12 and /or β-glucan on AD symptoms. The AD mice were divided into four groups: (a) mice receiving vehicle only (control), (b) mice receiving EC-12, (c) mice receiving β-glucan, and (d) mice receiving a mixture of EC-12 and β-glucan. AD symptoms were observed 5 weeks later after the first sensitization (n = 7).

**(a)**

| | |
|---|---|
| 0 | No petechial hemorrhage, crusting, or exfoliation on the back |
| 1 | Scattered petechial hemorrhages, crusts, or fine exfoliation |
| 2 | Scattered petechiae, crusting, and fine exfoliation on |
| 3 | Scattered petechial hemorrhages, crusting, and apparent keratin abrasion |
| 4 | Overall petechiae, crusting, exfoliation, and whitening |
| 5 | Generalized petechiae, crusting, keratin abrasion, and severe whitening |

**Dermatitis Clinical Score**

**(b)** **(c)**

Score 3 Score 5

**Representative skin lesions**

**(d)**

| Group | 0 | 1 | 2 | 3 | 4 | 5 | Score |
|---|---|---|---|---|---|---|---|
| (1) | 0 | 0 | 0 | 0 | 6 | 1 | 4.1±0.3 |
| (2) | 0 | 0 | 0 | 0 | 4 | 3 | 4.4±0.5 |
| (3) | 0 | 0 | 0 | 0 | 1 | 6 | 4.8±0.3 |
| (4) | 0 | 0 | 0 | 5 | 1 | 1 | *3.4±0.7 |

**Numbers having symptoms**

**Figure 3.** Effects of oral administration of EC-12 and/or β-glucan on AD Score. (a) shows symptoms to score and (d) shows the corresponding numbers to each score. The AD mice were divided into four groups: (1) mice receiving vehicle only (control), (2) mice receiving EC-12, (3) mice receiving β-glucan, and (4) mice receiving a mixture of EC-12 and β-glucan. According to the criteria for the dermatitis score shown in (a), the degrees of skins on the back were scored. The pictures show representative skin lesions with a score of 3 (b) and 5 (c). Data are presented as the mean ± SEM (n = 7). *p < 0.05, v.s. (1).

**(a)** **(b)**

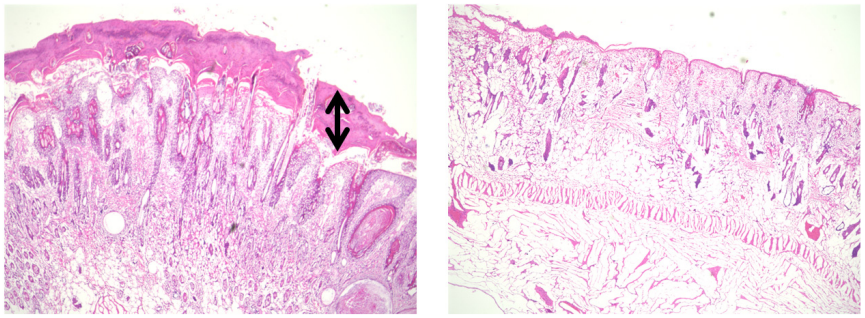

**Figure 4.** Effects of oral administration of EC-12 and/or β-glucan on AD histological symptoms. The AD mice were divided into two groups: (a) mice receiving vehicle only

and (b) mice receiving a mixture of EC-12 and β-glucan. The lesion on the back was surgically excised 5 weeks later after the first sensitization (n = 1). The arrow indicates thickening of the epidermis.

*3.2. A mixture of EC-12 and β-glucan reduces serum IgE levels*

IgE is considered to be the cause of itching in AD. The blood concentration of IgE is significantly higher in patients with AD compared to healthy subjects, so it is useful to examine IgE levels in the presence or absence of atopic factors. Therefore, we examined the ability of a mixture of EC-12 and β-glucan to reduce the amount of IgE in AD mice. An analysis of serum drawn on the last day of this schedule showed a significant reduction in the amount of IgE in AD mice administered with a mixture of EC-12 and β-glucan compared to control mice (Figure 5).

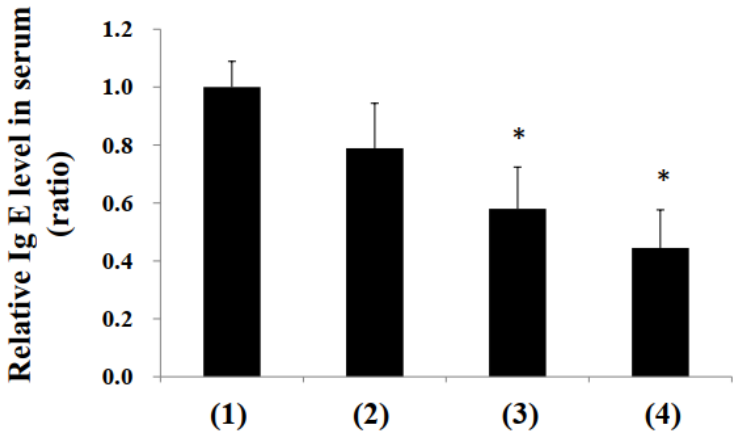


**Figure 5.** Effects of oral administration of EC-12 and/or β-glucan on IgE level of serum in AD mice. The AD mice were divided into four groups: (1) mice receiving vehicle only (control), (2) mice receiving EC-12, (3) mice receiving β-glucan, and (4) mice receiving a mixture of EC-12 and β-glucan. For this experiment, blood was collected 5 weeks late after the first sensitization. Data are presented as the mean ± SEM (n = 3). *$p < 0.05$, v.s. (1).

*3.3. A mixture of EC-12 and β-glucan suppresses the gene expressions of inflammatory cytokines*

Inflammatory cytokines are important in the pathogenesis of AD symptoms such as inflammation in the epidermis and dermis. Therefore, to investigate the mechanism involved in the reduction of disease incidence by a mixture of EC-12 and β-glucan, we examined the expressions of inflammatory cytokine genes such as IL-6, IL-1β, and TNF-α in the back skin. A gene expression analysis showed that the levels of inflammatory cytokines were reduced in the mice administered with a mixture of EC-12 and β-glucan compared to controls (Figure 6).

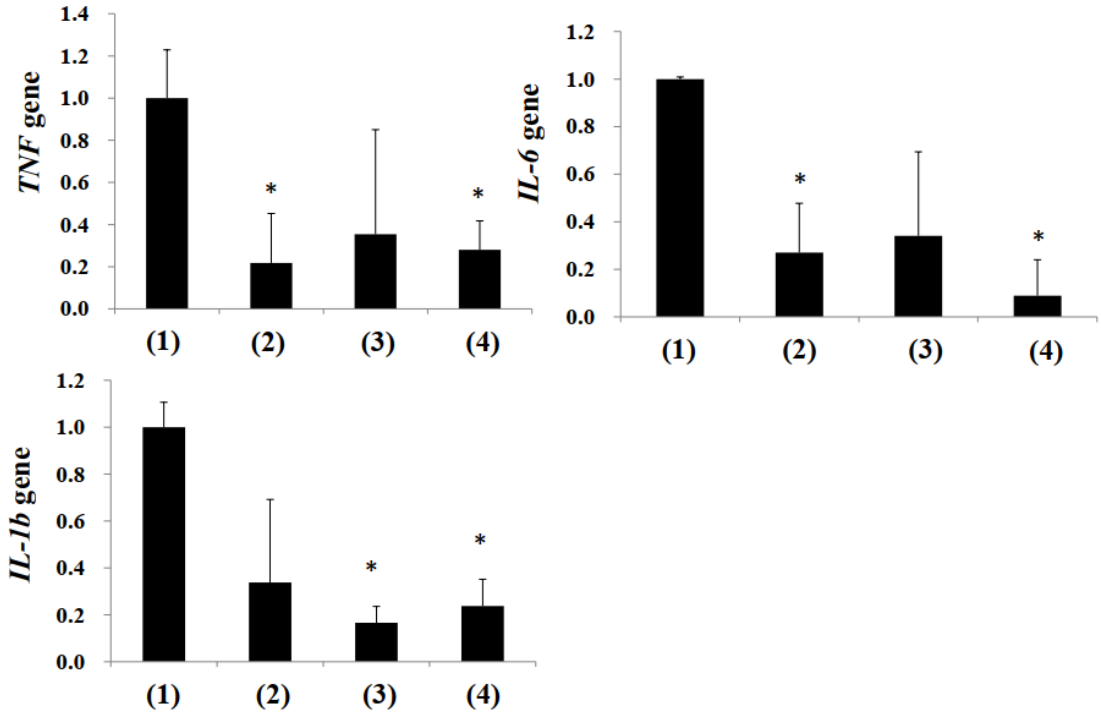


**Figure 6.** Effects of oral administration of EC-12 and/or β-glucan on the suppression of the inflammatory cytokine genes expressions in the back lesions of AD mice. The AD mice were divided into four groups: (1) mice receiving vehicle only (control), (2) mice receiving EC-12, (3) mice receiving β-glucan, and (4) mice receiving a mixture of EC-12 and β-glucan. For this experiment, the lesion on the back was surgically excised 5 weeks later after the first sensitization by DNFB. Data are presented as the mean ± SEM (n = 3). *$p < 0.05$, v.s. (1).

## 4. Discussion

Foods that exhibit immunostimulatory effects from a medical-preventive perspective or the widespread use of drugs that affect immune function are reported as functional foods [14]. Of note, EC-12 and/or β-glucan have shown various immunostimulatory effects.

AD is a chronic itchy eczema that repeatedly gets better and worse [15]. In AD, the function that protects the inside of the body from various external stimuli is weakened and the skin is inflamed [16]. Allergens and other stimuli from the outside are easier to enter, and these subsequently bind with immune cells and cause inflammation [17]. The beneficial effects of therapeutic steroids have been demonstrated in AD patients. After steroids enter cells, they bind to the glucocorticoid receptor (GR) and are translocated into the cell nucleus, where the complex regulates the expression of genes involved in inflammation, resulting in potent anti-inflammatory and immunosuppressive effects [18]. However, the main side effect of steroids is a weakening of the immune system, making the host more susceptible to infections. There have been few reports of side effects related to the ingestion of LAB and the daily use of LAB can alleviate various inflammatory diseases [19].

We previously reported that LAB supresses inflammatory diseases such as rheumatoid arthritis and collagen induced arthritis in mice [20]. In this study, we investigated the effects of the oral administration of EC-12 on AD in mice. The results indicated that the oral administration of EC-12 alone did not relieve skin swelling whereas a mixture of EC-12 and β-glucan was effective. The mixture of LAB and β-glucan is widely used when studying AD because it has various immunostimulatory effects [21]. No studies have clarified the effects of a mixture of EC-12 and β-glucan compared with their general individual use. Therefore, we orally administered either EC-12 or β-glucan alone or a mixture of EC-12 and β-glucan to AD mice and found that the mixture significantly relieved skin swelling compared with the use of each alone. Furthermore, the mixture of EC-12 and β-glucan significantly suppressed the production of allergy-related IgE and inflammatory cytokines compared with controls. These results suggest that the mixture of EC-12 and β-glucan strongly activates the immune system via a synergistic action compared to their use alone.

LAB, characterized by the production of large amounts of lactic acid from carbohydrates, suppresses inflammation [22]. Orally ingested LAB is recognized by epithelial cells lining the intestinal wall, macrophages, and dendritic cells (DC). LAB recognized by the receptors of intestinal epithelial cells has beneficial effects on the body by contributing to the control of cytokine production. However, LAB recognized by Toll-like receptors on macrophages and DC is taken up by M cells present in gut-associated lymphoid tissues and affects cytokine production and antibody production [23]. β-glucan is a polysaccharide produced from mushrooms, yeast, and grain, which exhibits various anti-inflammatory effects. β-glucan binds to dectin-1 expressed by DC and macrophages [24].

Allergen sensitization results in the production of IgE. External antigens that enter the body are taken up by antigen-presenting cells. Then, helper T cells that recognize antigen fragments are preferentially differentiated into Th2 cells that secrete cytokines to promote the production of IgE by B cells, which binds to the receptors of mast cells and basophils [25]. The cross-linking of receptors on mast cells and basophils induces the degranulation of these cells, releasing large amounts of histamine, a chemical messenger. These mediators induce vasodilation, vascular hyperpermeability, and provoke reactions in inflammatory diseases. In addition, activated mast cells and basophils induce inflammatory reactions by producing inflammatory cytokines and chemokines such as IL-6 and TNF-α [26].

In this study, a mixture of EC-12 and β-glucan that was orally administered suppressed the incidence of AD and decreased serum levels of IgE in AD mice. As mentioned above, EC-12 and β-glucan bind to different receptors. EC-12 and β-glucan suppress the activation of mast cells and basophils via different mechanisms, which might explain their co-operative activating effect. Furthermore, Th1 enhancement leads to the suppression of IgE responses and Th1 immune responses in AD dogs alleviated the symptoms of AD by improving the Th1/Th2 balance. Specific strains of LAB induced Th1 cell differentiation via an IL-12-dependent mechanism. The IL-12-inducing and Th1-enhancing effects of many LAB strains have been reported. β-glucan also activates Th1 responses via IFN-γ production. When the activation of Th1 cells becomes dominant over Th2 cells involved in allergy, IgE production is suppressed.

The search for novel probiotic strains and their effectiveness are required in order to advance the functional research of probiotics represented by LAB. Human clinical trials in drug development should be referenced, but more discussion is needed to verify the efficacy of probiotics. This study

will lead to the clues to increase the understanding of the various preventive effects caused by a mixture of bacteria and glucan in different inflammatory diseases.

## 5. Conclusions

Our study demonstrates that the oral administration of EC-12 and β-glucan significantly decreased the incidence and attenuated the symptoms of AD by reducing the level of IgE in the dorsal skin compared to either EC-12 or β-glucan. Furthermore, the combination reduced the expression level of inflammatory cytokines such as IL-6 and IL-1β, which was closely correlated with the preventive effects of EC-12 and β-glucan on AD. Our results suggest a novel potential effect of the combination of EC-12 and β-glucan on the reduction of IgE and inflammatory cytokines, which might exert a preventive or attenuative effect for patients with autoimmune diseases, including AD.

## Use of AI tools declaration

The authors declare they have not used Artificial Intelligence (AI) tools in the creation of this article.

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## Conflict of interest

The authors declare no conflicts of interest associated with this manuscript.

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