Original Article

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Dietary supplementation with *Nopalea cochenillifera* enhances fecal mucin production and modulates serum immunoglobulin levels in a dose- and time-dependent manner in BALB/c mice

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SUMMARY: The aim of this study was to determine the time-dependent effects of the prickly pear (*Nopalea cochenillifera*) on the intestinal environment and immune function in BALB/c mice, which exhibit a predominant T helper 2 immune response. Five-week-old female BALB/c mice were divided into a control group and groups fed diets supplemented with 5% or 10% *N. cochenillifera* powder (NCP). Blood and feces were collected every 7 days. On day 28, in addition to blood and feces, the small intestine, cecum, and large intestine were collected. The cecum content weight, cecum content pH, and fecal mucin were examined. Serum antibody levels (total Immunoglobulin (Ig)M) were lower on days 14 and 21 in the 10% NCP group than in the control and 5% NCP groups. Total serum IgG levels were higher at all time points and total IgA levels were higher on days 7, 14, 21 and 28 in the 5% NCP and 10% NCP groups than in the control group. Total fecal IgA levels were lower in the 5% NCP and 10% NCP groups than in the control group from 14 days. Fecal mucin contents were higher in the 10% NCP group than in the control group from 7 days. These results suggest that *N. cochenillifera* supplementation exerts time-dependent effects on serum antibodies and the intestinal environment in BALB/c mice. In particular, NCP at 10% may enhance the intestinal mucosal barrier function and promote systemic immune responses. Further mechanistic studies are needed to determine the effects on immune responses.

Keywords: cactus, immune function, intestinal environment, immunoglobulins, mucin, mice

1. Introduction

The immune system consists of a diverse and complex system responsible for defense against a wide variety of pathogens and is essential for protecting the body from disease. The immune system comprises innate and adaptive immunities, which are composed of specific immune components that suppress infection. Immune function is influenced not only by genetic factors, but also by aging, lifestyle, and environmental factors related to susceptibility to disease-causing agents. Thus, weakened immune function can lead to autoimmune diseases, inflammatory diseases, and cancer (*1*). Chemical compounds in certain foods have been shown to regulate signaling and cellular phenotypes, ultimately affecting pathophysiology. Previous studies have reported that immune cells are activated when functional foods, such as *Chlorella* (*2,3*), probiotics, such as *Lactobacillus plantarum* (*4*), and prebiotics, such as guar gum and inulin (*5-7*), are consumed.

There is a growing need to develop and increase the production of various food products to address the threat of food shortages caused by global population growth. The food industry is trying to meet the standards of the Sustainable Development Goals, which aim to provide a stable supply of food containing a variety of nutrients; however, meeting these nutritional goals is proving to be a challenge. In addition, there is an urgent need to search for new products and to study their functional effects. The stem nodes of prickly pears are used as food in more than 30 countries, and in 2017, the United Nations Food and Agriculture Organization lauded prickly pear as a crop that could save the world from a food crisis. The family Cactaceae includes more than 1,450 species in about 30 genera and is divided into four subfamilies: Peireskioideae, Maihuenioideae, Opuntioideae, and Cactoideae (*8*). The subfamily Opuntioideae includes about 20 genera and 300 species, several of which

are used for food. Kasugai city in Aichi Prefecture is Japan's largest producer of seeding cactus and is using cactus to revitalize the financial and economic status of the city. Cactus is a useful vegetable that can be grown under a variety of conditions owing to its vitality, ability to thrive under different environmental regimes, and cultivation characteristics, and it has attracted attention as a self-sufficient agricultural product and food source because it is easy to grow in the Japanese climate. Furthermore, some varieties of cacti, such as prickly pear, have beneficial effects, including antioxidant effects (*9*) and regulatory effects on glucose (*10-13*) and lipid metabolism (*14*). Cactus is high in dietary fiber and various minerals (*15,16*). Although dietary fiber is a carbohydrate, most of it reaches the large intestine undigested because it cannot be broken down by digestive enzymes secreted by the human digestive tract (*17*). Dietary fiber is a potential prebiotic, and it undergoes partial or complete fermentation by the microflora in the colon, the products of which are a major source of energy for the intestinal microflora. The breakdown products of fiber are also important for the maintenance of the colonic epithelium (*18*). When food-derived, soluble dietary fiber and non-digestible polysaccharides undergo fermentation by the microflora in the gut, short-chain fatty acids (SCFAs) are produced, resulting in a decrease in pH, and they are involved in various cellular functions, including gene expression, chemotaxis, differentiation, proliferation, and apoptosis, thereby affecting the physiological functions of the host (*19,20*). Therefore, the fiber-rich prickly pear may have an effect on immune function. However, research has focused on large prickly pear (*Opuntia ficus-indica*), with relatively few studies of small prickly pear (*Nopalea cochenillifera*).

We have previously demonstrated that *N. cochenillifera* regulates innate and adaptive immune functions *via* the gut microflora in C3H/HeN mice, which exhibit a predominant T helper (Th) 1 immune response (*21*). In experiments focusing in the adaptive immune system, Th2-dominant mice, such as BALB/c mice, are commonly used. Changes in the intestinal environment, gastrointestinal tract, and systemic immune system due to continuous consumption of *N. cochenillifera* have not been studied sufficiently. In this study, we examined the time-dependent effects of *N. cochenillifera* on the intestinal environment and immune function in BALB/c mice, which exhibit a predominant Th2 immune response.

2. Materials and Methods

2.1. *N. cochenillifera* sample

N. cochenillifera cladodes were grown in a greenhouse in Chubu University in Aichi Prefecture, Japan. Young cladodes were harvested from plants aged 3 years. Harvested cladodes were cut into smaller pieces, freeze-dried, and milled for use in the following experiments.

2.2. Animals and diets

Six-week-old female BALB/cCrSlc mice (15–20 g) were obtained from Japan SLC (Hamamatsu, Shizuoka, Japan) and housed at 24 ± 3°C and 55 ± 10% relative humidity with a 12 h light-dark cycle in a specific pathogen-free facility. After acclimatization for 5 days, the mice were provided with the experimental diets and water *ad libitum*. Mice were divided into three groups: control (n = 8), 5% *N. cochenillifera* powder (NCP) (n = 8), and 10% NCP groups (n = 8). Mice were housed in groups of four per cage. The diets were prepared according to the recommendation of the American Institute of Nutrition and AIN-93G rodent diet (*22*). The control group received AIN-93G as a control diet and the 5%NCP and 10%NCP groups received AIN-93G supplemented with 5% NCP or 10% NCP, respectively (Table 1). The mice consumed the diets for 4 weeks. The experimental design was in accordance with the guidelines for animal experimentation and was approved by the Animal Experimental Committee of the Chubu University (authorization number: 202110051).

2.3. Animal protocol

Blood and fecal samples were collected every week. The serum was separated by centrifugation at 700× g for 15 min, collected, and stored at −20°C until analyses. Feces were collected on days 7, 14, 21, and 28. The feces were stored at −20°C, freeze-dried, and milled. At the end of the feeding period, the mice were anesthetized with isoflurane and the liver, spleen, small intestine, large intestine, and cecum were removed. The liver, spleen, and cecal contents were weighed, and the small and large intestines were measured for length. Cecal contents were measured for pH value.

2.4. Analyses of fecal total Immunoglobulin (Ig) A and mucin

Total IgA and mucin assays were performed as described previously (*21*). Total IgA concentrations in feces were

Table 1. Composition of the test diets (%)

Component (%)	Control	5%NCP	10%NCP
Cornstarch	63.2	5.82	53.2
Casein	20	20	20
Soy bean oil	7	7	7
cellulose powder	5	5	5
Mineral mix(AIN-93G-MX)	3.5	3.5	3.5
Vitamin mix(AIN-93G-VX)	1	1	1
L-Cystine	0.3	0.3	0.3
tert-Butylhydroqunone	0.0014	0.0013	0.0042
N. cochenillifera	0	5	10

measured using an enzyme-linked immunosorbent assay (ELISA) kit for quantitative analyses (Invitrogen, Waltham, MA, USA). Mucin levels were quantified using a fluorometric assay for quantitative analysis of fecal mucin (Cosmo Bio Co., Ltd., Koto Ward, Tokyo, Japan). Fecal total IgA and mucin levels were measured in accordance with the manufacturers' instructions.

2.5. Analyses of serum total IgM, IgG, and IgA

The total IgM, IgG, and IgA concentrations in serum were measured using an enzyme-linked immunosorbent assay (ELISA) kit for quantitative analyses (Invitrogen, Waltham, MA, USA).

2.6. Statistical analysis

Data are expressed as the mean ± SEM. All statistical analyses were performed using GraphPad Prism v10.2.3. (GraphPad Software, San Diego, CA, USA). For data that followed a normal distribution, two-way ANOVA was performed. For cecal content weight and pH, one-way ANOVA was used. For serum IgA, IgG and IgM, as well as fecal mucin and IgA, repeated measures ANOVA was performed. Data that did not follow a normal distribution were analyzed using the nonparametric Kruskal-Wallis test (one-way ANOVA). A Tukey's post hoc test was performed when a significant effect was determined by two-way ANOVA. A Games-Howell post hoc test was conducted when a significant effect was found by the Kruskal-Wallis test. Differences were considered significant when $p < 0.05$.

Table 2. Tissue and body weights and lengths of small and large intestines

	Control	5%NCP	10%NCP
Daily food intake (g)/mouse/day	2.37 ± 0.05	2.61 ± 0.09	2.49 ± 0.06
Body weight (Before)	16.3 ± 0.3	16.3 ± 0.3	16.4 ± 0.3
Body weight (28 days)	18.3 ± 0.2	18.9 ± 0.2	18.6 ± 0.4
Liver (g)	0.741 ± 0.021	0.793 ± 0.014	0.695 ± 0.039
Spleen (g)	0.102 ± 0.003	0.106 ± 0.0003	0.096 ± 0.003
Small intestine (cm)	37.3 ± 0.2	37.3 ± 0.7	38.3 ± 0.5
Large intestine (cm)	8.2 ± 0.2	8.2 ± 0.2	7.9 ± 0.2

Data are reported as the mean ± SEM ($n = 8$). *$p < 0.05$.

3. Results

3.1. Tissue and body weight and small and large intestine lengths

The body weight, tissue weight, and lengths of the small and large intestine on the 28th day of the experiment are shown in Table 2. There were no significant differences among groups in body weight, liver, and spleen weight or the length of the small and large intestine.

3.2. Dry fecal weight, cecum weight, and pH of cecal contents

To determine the effects of *N. cochenillifera* intake on the intestinal environment, the dry weight of the feces collected every 7 days and the contents of the cecum on day 28 were evaluated (Figure 1). The dry weight of feces was higher than that in the control group from day 7, with a 1.3-fold increase in the 5% group and a 1.5-fold increase in the 10% group over the total experimental period (Figure 1A). There was no significant differences in the weight of the cecal contents among the three groups (Figure 1B), and the pH values of the cecal contents in the groups given *N. cochenillifera* were significantly lower than those of the control group (Figure

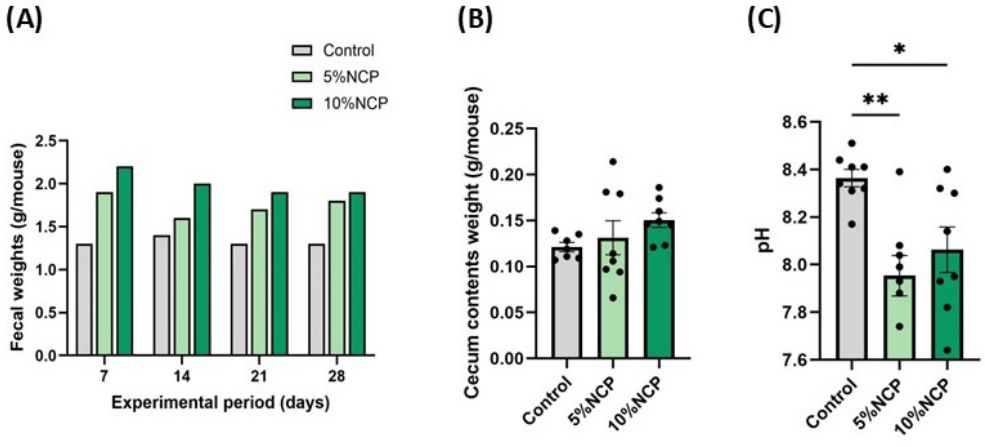

Figure 1. Dry weight of feces, cecal content weight, and pH of the cecal contents in mice fed a *Nopalea cochenillifera* diet. Dry fecal weight (**A**), weight of cecal contents (**B**), and pH of cecal contents (**C**) obtained in each 7-day period after 28 days in the control group, 5% group, and 10% group are shown. Data are expressed as the mean ± SEM ($n = 8$). *$p < 0.05$, **$p < 0.01$.

1C, $p < 0.05$).

3.3. Intestinal IgA and secreted mucins in mice fed *N. cochenillifera*

To determine the effects of *N. cochenillifera* intake on the gastrointestinal immune system, total IgA and mucin contents in feces were measured (Figure 2). Total fecal IgA was significantly lower in the 5% NCP group on days 14, 21 and 28 and in the 10% NCP group on days 14 and 28 compared with those in the control group (Figure 2A, $p < 0.05$). The total mucin content in the feces was significantly higher on day 7, 14 and 28 in the 5% NCP group and on days 7, 14, and 21 in the 10% NCP group compared with those in the control group (Figure 2B, $p < 0.05$).

3.4. Antibody concentrations in the serum

To investigate the immunostimulatory effects of *N. cochenillifera* intake on the systemic immune system, total serum IgM, IgG, and IgA were measured (Figure 3). The IgM levels in the NCP 10% group on days 14 and 21 were significantly lower than those in the control groups (Figure 3A, $p < 0.05$). Additionally, the IgG and IgA levels in the NCP 5% and NCP 10% groups on days 7, 14, 21 and 28 were higher than those in the control group. Furthermore, it was observed that both

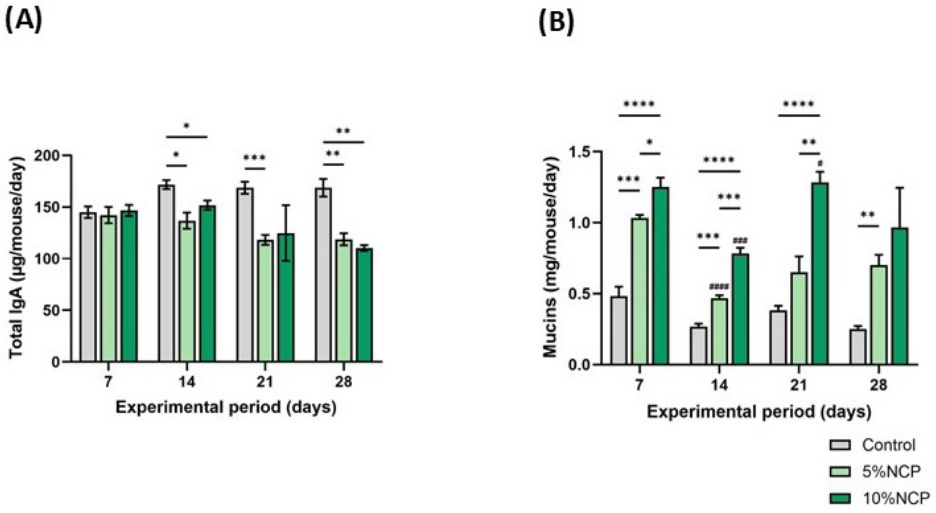

Figure 2. Effect of a diet containing *Nopalea cochenillifera* on total fecal IgA and mucin levels in mice. Amounts of total fecal IgA (**A**) and mucins (**B**) obtained over each 7-day period from the control group, NCP5%, and NCP10% are shown. Data are expressed as the mean ± SEM ($n = 6$). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, significant difference was observed between the groups. $^{###}p < 0.001$, $^{####}p < 0.0001$, significant difference was observed compared to the previous week.

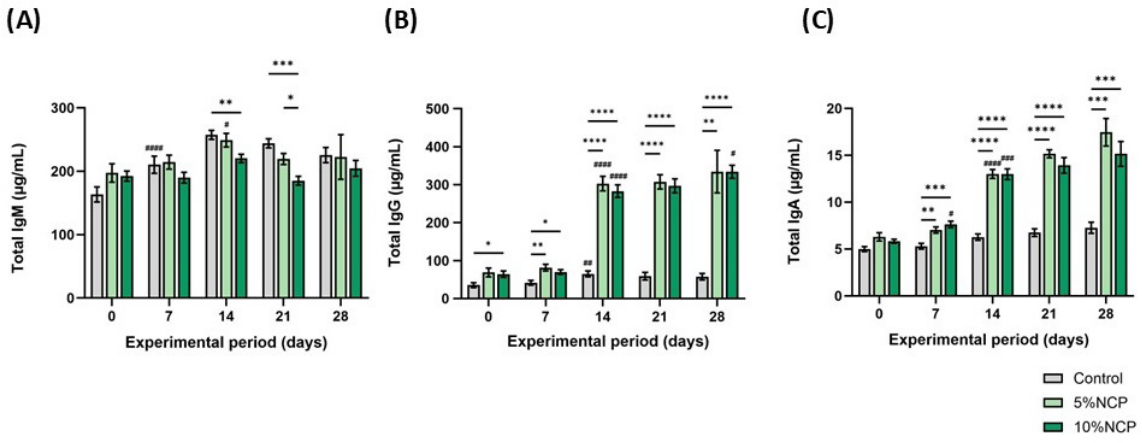

Figure 3. Effects of a diet containing *Nopalea cochenillifera* on the production of total antibodies in serum from mice. The IgM (**A**), IgG (**B**), and IgA concentrations (**C**) in the serum obtained after each 7-day period from the control group, NCP5%, and NCP10% are shown. Data are expressed as the mean ± SEM ($n = 8$). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, significant difference was observed between the groups. $^{#}p < 0.05$, $^{##}p < 0.01$, $^{####}p < 0.0001$, significant difference was observed compared to the previous week.

had increased on day 14 compared to the previous week (Figure 3B, 3C, $p < 0.05$).

4. Discussion

In this study, we examined the effects of dietary supplementation with *N. cochenillifera* on antibody production and intestinal immune function in BALB/c mice, revealing novel functions by which the species improves the intestinal environment, intestinal barrier function, and systemic immune function in mice.

We have previously shown that approximately 50% of NCP consists of dietary fiber, of which about 80% is insoluble and 20% is soluble (*21*). Furthermore, dietary fiber in cactus is rich in cellulose, hemicellulose, and pectin (*15,16*). Cellulose, hemicellulose, and other insoluble fiber have various intestinal regulatory effects, including roles in softening stool, physically stimulating the intestinal tract, and increasing stool bulk, due to water absorption and the consequent increase in volume of the stool (*23*). Some soluble fibers, such as pectin, inulin, and guar gum, exert prebiotic effects; however, like insoluble fiber, they have large molecular sizes and are not as rapidly digested as simpler carbohydrates and sugars by intestinal bacteria (*24*). On this basis, we hypothesized that the dietary fiber-rich *N. cochenillifera* would function as a modulator of the intestinal environment. In the present study, *N. cochenillifera* consumption increased fecal weight and deceased the pH of cecal contents. Probiotic *Bifidobacteria* produces the SCFAs acetic acid and lactic acid, whereas butyric acid is produced by bacteria such as *Clostridium* and *Ruminococcus*. It is thought that enteric bacteria that produce putrefactive products that are not beneficial to the host are generally susceptible to low pH, and increased SCFAs production will limit the growth and activity of intestinal bacteria (*25*). As reported earlier, *N. cochenillifera* consumption decreases succinate and lactic acid in the cecum, increases butyrate, and lowers fecal pH. The decrease in pH in the cecal contents was likely due to fiber digestion and consequent SCFAs production by intestinal bacteria, which may have promoted intestinal regulation. The regulatory effect on the intestine was observed from the 7 days of intake, as indicated by the increase in the amount of feces. The fecal weight also increased with *N. cochenillifera* consumption, which may be attributed to its insoluble fiber. These findings suggested that both soluble and insoluble fiber in *N. cochenillifera* affect the intestinal environment.

Next, the total IgA concentration and mucin content in feces were measured to evaluate the barrier function of the gastrointestinal tract after supplementation with *N. cochenillifera*. The gastrointestinal tract is a unique tissue that is exposed not only to the threat of invasion by pathogenic microorganisms but also to the constant presence of foreign substances consumed in daily life or produced by intestinal bacteria. Therefore, immune cells in the gut lamina propria have different functions from those in the lymph nodes and spleen. The lymphoid tissue of the gut also has unique functions. Because the mucin layer exhibits a barrier function that prevents the passage of intestinal bacteria to the inside of the body (*26*) and IgA prevents bacterial contact with epithelial cells (*27*), mucin and IgA may be involved in maintaining intestinal health. Furthermore, the secretion of mucin and IgA is stimulated by SCFAs containing butyrate (*28,29*). We have reported that *N. cochenillifera* consumption increases butyrate levels in the cecum (*21*). These findings suggest that the production of fecal mucin resulting from *N. cochenillifera* consumption may be influenced by SCFAs produced by intestinal bacteria. In our study, the mucin content in feces increased significantly after the first week of *N. cochenillifera* consumption. In contrast to expectations, total fecal IgA decreased after the second week. We have previously shown that fecal mucin and IgA are increased in mice fed *N. cochenillifera* for 6 weeks. However, in the present study, similar results were not obtained for fecal IgA. This discrepancy could be attributed to differences in the mouse strain, duration of the experiments, and nature of comparisons. Although mechanism was not clarified in this study, it may involve polymeric immunoglobulin receptor (pIgR) expressed on the basement membrane of epithelial cells, as pIgR has been reported to be involved in the transcytosis of secretory IgA from inside the cells to the lumen of the gastrointestinal tract (*30*). *N. cochenillifera* may suppress the expression of pIgR, reducing IgA release into the lumen.

In further analyses of the systemic immunostimulatory effects of *N. cochenillifera* supplementation, serum IgM, IgG, and IgA concentrations were measured. Total serum IgM decreased after the 14 days in mice fed the experimental diet; in contrast, total IgG and IgA levels were significantly higher than those for the control diet. These results indicate that of the three Ig classes, *N. cochenillifera* may have the greatest effects on the production of IgG, followed by IgA. IgM is produced by B cells and is found primarily in blood. The increases in serum IgG and IgA suggest a potential contribution to enhanced immunity and infection prevention. Interestingly, IgG and IgA increased during the same period of time that IgM decreased, which may indicate that *N. cochenillifera* promoted a class switch from IgM to IgG and IgA. Although we were unable to clarify the detailed antibody production capacities in this study, an increase in antibody production in response to soluble dietary fiber has been observed in feeding experiments; however, a similar increase is not observed when lymphocytes are directly treated with fiber (*5*). Accordingly, the observed effects may be indirectly mediated by the intestinal microflora and immunomodulatory function of soluble dietary fiber. The increases in serum IgG and IgA following

N. cochenillifera consumption suggest that the species contributes to enhanced immunity and infection prevention.

In conclusion, we showed that supplementation with *N. cochenillifera* results decreases the pH of the cecal contents and increases the amount of feces and fecal mucin in mice. Furthermore, we found significant increases in total serum IgG and IgA after supplementation with *N. cochenillifera*. These findings suggest that *N. cochenillifera* has potential as a prebiotic and may modulate immune function.

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Conflict of Interest: The authors have no conflicts of interest to disclose.

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