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# Administration of *N*-acetyl-D-glucosamine Thickens the Mucin Layer and Induces Flora Diversity in the Intestinal Tract of Elderly Mice

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**Abstract.** *N*-acetyl-D-glucosamine (GlcNAc) is present as a constituent carbohydrate of mucins, which are secreted from specific mucin-producing epithelial cells in the intestinal tract. Here we administered GlcNAc to an aging mouse model and verified its effectiveness on intestinal mucins and flora as compared with D-glucosamine hydrochloride (GlcN·HCl). Administration of GlcNAc was found to change the concentration of organic acids in the intestinal feces and to thicken the mucin layer. In intestinal epithelial cells of mice given GlcNAc, the expression level of a gene involved in synthesis of the mucin-type sugar chain (Core 2 β 1,6-*N*-acetylglucosaminyltransferase-2; C2GnT2) was upregulated. In addition, an increasing diversity of intestinal flora was observed in the feces of mice given GlcNAc. These improvements were more pronounced after GlcNAc administration than after GlcN·HCl administration. In conclusion, administration of GlcNAc improves the intestinal mucin layer and flora in elderly mice.

**Keywords:** *N*-acetyl-D-glucosamine (GlcNAc), intestinal mucin layer, intestinal flora, Core 2 β 1,6-*N*-acetylglucosaminyltransferase-2 (C2GnT2).

## 1. Introduction

*N*-acetyl-D-glucosamine (GlcNAc) is a type of amino-sugar that widely occurs in nature and plays an important role in biological processes. In the human body, GlcNAc comprises the major part of glycosaminoglycans and constitutes various glycoconjugates such as glycoproteins and glycolipids, which are important in biological functions.

Mucins cover the trachea and gastrointestinal tract and form a gel layer to protect the epithelia from pathogenic bacteria and viruses, digestive enzymes, and acidic digestive juices. They are large glycoproteins with molecular weights ranging from 0.5 to 20 MDa, and are highly glycosylated by ~80% carbohydrates, which comprise mainly mucin-type oligosaccharides (*O*-glycans) [1]. The addition of mucin-type oligosaccharides to a protein starts with covalent linkage of *N*-acetyl-D-galactosamine (GalNAc) to the hydroxyl group of a Ser or Thr residue in a protein (Tn antigen). After that, Galactose (Gal), GalNAc and GlcNAc are each added to the sugar chain one by one, and the extended sugar chain often ends with L-Fucose or Sialic acid. Mucin-type oligosaccharides have eight proposed core-type structures (Table 1), and these generally exist in the state of the sugar chain that extends in addition to the core structure [2].

During aging, secretion of mucin decreases and the thickness of the mucin layer is reduced [3]. The mucin layer helps to provide a barrier against pathogenic bacteria in the intestinal tract, and thus a reduction of the mucin layer is regarded to increase the risk of disease [4]. Furthermore, intestinal mucin is involved in fixing the intestinal flora, and a reduction of the mucin layer is also thought to affect these bacteria.

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The intestinal tract of breast-fed infants is rich in Bifidobacteria known as probiotics, whereas *Escherichia coli* (*E. coli*) and *Clostridium perfringens* (*C. perfringens*) are barely observed. During aging, Bifidobacteria decrease in content, whereas *C. perfringens*, *E. coli* and other bacteria increase significantly, and this change in intestinal flora is thought to lead to a progression of intestinal disease [5].

Intestinal bacteria have various glycosidases that play an important role in digesting and utilizing mucin-type oligosaccharides [6]. Indeed, glycosidases from several intestinal bacteria have been shown to be involved in mucin digestion by analyses of their enzymatic properties [7], [8]. The decrease in the mucin layer due to aging is considered to change the composition of intestinal flora and is thought to be a factor that Bifidobacteria is difficult to fix [9].

Table 1: Core structures of mucin-type oligosaccharide

| Type | Structure |
|---|---|
| Tn antigen | GalNAc 1-Ser/Thr |
| Core 1 | Gal 1-3GalNAc 1- Ser/Thr |
| Core 2 | Gal 1-3(GlcNAc 1-6)GalNAc 1- Ser/Thr |
| Core 3 | GlcNAc 1-3GalNAc 1- Ser/Thr |
| Core 4 | GlcNAc 1-3(GlcNAc 1-6)GalNAc 1- Ser/Thr |
| Core 5 | GalNAc 1-3GalNAc 1- Ser/Thr |
| Core 6 | GlcNAc 1-6GalNAc 1- Ser/Thr |
| Core 7 | GalNAc 1-6GalNAc 1- Ser/Thr |
| Core 8 | Gal 1-3GalNAc 1- Ser/Thr |

We have focused on GlcNAc as a constituent of mucin-type oligosaccharides. The aim of this study was to determine the effects of the administration of GlcNAc on the intestinal mucins and flora of the elderly by using two types of elderly model mouse: namely, senescence accelerated mice (SAMP/1 mice) and natural aging mice (ICR mice). After preliminary feeding, the mice were forcibly given carbohydrate for 8 weeks, and the effect on the intestinal mucin layer and flora was investigated. We also examined epithelial cells for expression of C2GnT2, a glycosyltransferase involved in synthesis of the mucin-type oligosaccharides that adds GlcNAc to the Core 1 and Core 2 structures to synthesize Core 2 and Core 4 structures. Furthermore, we also examined the effects of D-glucosamine hydrochloride (GlcN·HCl), the GlcNAc-derivative.

## 2. Materials and Methods

### 2.1. Experimental Animal and Breeding Environment

Fifteen male 15-week-old aging-accelerated mice (SAMP/1 mouse; Japan SLC, Inc., Hamamatsu, Japan), and 15 male ICR mice aged 15 months were used in the study. ICR mice were kindly gifts from Dr. Suda (Department of Food, Agriculture and Environment, Miyagi University, Sendai, Japan).

Before carbohydrate administration, SAMP/1 mice and ICR mice were preliminarily fed until they reached the appropriate age for the experiment. In brief, SAMP/1 mice were fed for 3 months and ICR mice were fed for 6 months with free access to water and feed (MR lab stock; Nosan Co., Ltd., Kanagawa, Japan), whose nutritional contents were 3.9% fat, 18.8% protein, 54.7% carbohydrate, and 6.6% dietary fiber. From the start of the experiment, the amount of feed that the mice ate and the amount that remained were measured once weekly. The mice were individually housed in cages under a 12h/12h light/dark cycle, with a temperature of 20~26 ℃ and a humidity of 40~60%; the flooring was replaced once a week.

Carbohydrate administration to the SAMP/1 mice was allocated as follows: group 1, distilled water (DW) (n=5); group 2, GlcN·HCl (n=5); and group 3, GlcNAc (n=5). Carbohydrate administration to the ICR mice was the same as that for the SAMP/1 mice. The body weight of the mice was measured by using an electronic balance. Each mouse was placed in a box-shaped gauge to prevent it moving around on the electronic balance. Feed intake was determined by subtracting the remaining feed from the feed dosage.

### 2.2. Method of Administration and Preparation of the Administration Feed

The dose of GlcN·HCl and GlcNAc for each mouse was calculated on the basis of its body weight relative to a dose of 1,000 mg of GlcNAc per day in humans or 1,500 mg of GlcN·HCl per day in humans, and under the assumption of a human body weight of 62 kg, which is average weight of a man 65~70 years old. The carbohydrates was dissolved in DW, and stored at 4 ℃ until use.

A 100 μL aliquot of the two prepared types of sugar solution (GlcN·HCl and GlcNAc) was measured into a 1 mL syringe, and forcibly administered to the mouse stomach by a gastric tube needle (Shimadzu Corp., Kyoto, Japan). Administration was carried out once a day at 12 pm for 8 weeks.

## 2.3. Sample Collection

Eight weeks after the start of carbohydrate administration, the mice were sacrificed by cervical dislocation following anesthetization using 100 μL of Somnopentyl® (Kyoritsu Seiyaku, Tokyo, Japan).

Part of the large intestine, which was not washed with PBS, was embedded in Optical Cutting Temperature compound® (OCT compound, Sakura Fine Tech Japan Co., Ltd., Tokyo, Japan), and slices of the compound were frozen in dry ice plus hexane. Another part of large intestine was dipped in RNAlater® (Life technology, MA, USA) and stored in −30 ℃ for RNA sample preparation (see below).

The feces were gathered from the large intestinal tract directly into a 1.5 mL tube. One sample was stored at −30 ℃, while another sample was weighed to 0.3 g and suspended in 600 μL of DW for organic acid analysis (see below).

## 2.4. Analysis of Tissue Sections

For mucin layer measurement, slices of the intestine compound were dyed by Periodic Acid Schiff (PAS), which is commonly used for polysaccharide detection. By PAS staining, simple polysaccharides, neutral and acidic mucus proteins, and lectins are stained red, whereas acidic mucus polysaccharides and neutral mucus polysaccharides are stained purple.

The stained sections were observed under an optical microscope (Olympus BX40). Images were taken (Axio version Rel.4.6), and the mucin layer was measured in 20 places per sample. Thus, the thickness of the mucin layer in one sample was an average value of 20 measurements.

## 2.5. Organic Acid Concentration of Mouse Intestinal Feces by HPLC Analysis

The 0.3 g samples of feces in 0.6 mL of DW were centrifuged (31,800 × g, 10 min, 4 ℃), and the supernatant was added to 1/10 volume of 14% perchloric acid, and then re-centrifuged (31,800 × g, 10 min, 4 ℃). For purification, the resulting supernatant was filtered by Cosmo 0.45 μm nice filter W (water-based: Nacalai Tesque, Kyoto, Japan), and degassed under reduced pressure to remove the carbon dioxide. Next, 5 μL aliquots of the samples were subjected to HPLC analysis.

The organic acid analysis HPLC system comprised an LC-10AD pump (Shimadzu Corp., Kyoto, Japan), Waters 431 electrical conductivity meter, Waters organic acid column (7.8 mm × 30 cm × 2 pcs), column heater module, and system controller. The mobile phase was 5 mM *p*-toluenesulfonic acid solution. The reaction solution was 5 mM *p*-toluenesulfonic acid solution, 20 mM Bis-Tris, and 100 μM EDTA (free acid). Isocratic elution was used with a flow rate of 0.8 mL/min. The column temperature was set at 45 ℃. Detection was via an electrical conductivity meter with a base voltage of 2000 mV and sensitivity of 0.01 mV. Components were identified via the CBM-20A data module (Shimadzu).

## 2.6. Organic acid T-RFLP Flora Analysis of Mouse Intestinal Feces

The stored (−30 ℃) fecal samples of SAMP/1 mice and ICR mice were thawed at room temperature and weighed to obtain 100 mg samples.

Terminal restriction fragment length polymorphism (T-RFLP) flora analysis was carried out according to the method of Nagashima *et al.* [10], using the following two primers: 5'-HEX-labeled 516f (5'-TGCCAGCAGCCGCGGTA-3'; *E. coli* positions 516 to 532) and 1510r (5'-GGTTACCTTGTTACGACTT-3'; *E. coli* positions 1510 to 1492). The PCR products were digested by *Bsl*1. Fragment analysis was carried out by using an ABI PRISM 313 DNA Sequencer (Applied Biosystems, CA, USA). The length of each fragment was determined by using operational taxonomic units (OTUs). Cluster analysis was carried out by the analysis software Gene Maths (Applied Maths, St-Martens-Latem, Belgium). The clustering of patterns was calculated by the pearson correlation and the unweighted-pair group method using average linkages (UPGMA).

The peak in the T-RFLP data of each sample was assigned to the OTU. The group speculated from the OTU was based on the mouse intestinal flora database. The OTU peak area ratio of each sample was calculated to obtain an average value, which was then compared with the other test groups.

The experiment was repeated at least three times. The results were reported as the mean ± SEM. Significant differences between the groups were tested by Student's t-test.

## 2.7. Quantitative Expression Analysis by Real-time PCR

We performed two-step real-time quantitative PCR to characterize the expression of C2GnT2 mRNA in the intestinal epithelial cells of mice. Total RNA was isolated from the large intestine stored in RNAlater® by using TRIzol reagent (Invitrogen, CA, USA). All cDNAs were synthesized by using a Quantitect Reverse Transcription Kit (Qiagen, Tokyo, Japan) according to the manufacturer's recommendations. Real-time quantitative PCR (RT-PCR) was carried out with an Applied Biosystems Real-time PCR System 7300 (Applied Biosystems, Warrington, United Kingdom) and Platinum SYBR Green qPCR SuperMix-UDG (uracilDNA glycosylase) with ROX (6-carboxyl-X-rhodamine) (Invitrogen).

The primers used for analysis of C2GnT2 and β-actin are shown Table 2. The PCR cycling conditions were 5 min at 50 ℃, 5 min at 95 ℃, and then 40 cycles of 15 sec at 95 ℃, 30 sec at 60 ℃, and 30 sec at 72 ℃. The reaction mixtures contained 2.5 μL of sample cDNA diluted sixfold and 7.5 μL of master mix, which included the sense and antisense primers. Expression of β-actin was assessed in each sample and used as an internal control to normalize differences between samples and to calculate relative expression levels.

Table 2: Sequences of primers used in this study

| | | Structure |
|---|---|---|
| -actin | Sense primer | GATGACGATATCGCTGCGCTG |
| | Antisense primer | GTACGACCAGAGGCATACAGG |
| C2GnT2 | Sense primer | ATTGCGTACTCCATGGTGGTG |
| | Antisense primer | CATACTGTTCTGCCCTTTCA |

# 3. Results and Discussion

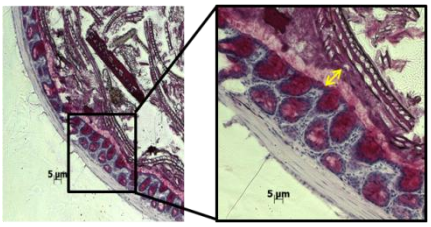
## 3.1. Body Weight and Food Consumption in SAMP/1 Mice and ICR Mice

Among the SAMP/1 mice, two died in each group, and the experiment was carried out on the remaining three mice. Among the ICR mice, one died during DW administration and GlcNAc administration; the experiment was conducted on the remaining four mice in these groups and on five mice in the GlcN ·HCl group.

In terms of body weight and feed intake, there were no large differences among the types of carbohydrate administration. The body weights of the SAMP/1 mice were 28.6~32.6 g (DW), 29.5~35.1 g (GlcN ·HCl), and 30.0~33.1 g (GlcNAc). The body weights of the ICR mice were 47.4~50.0 g (DW), 43.4~48.4 g (GlcN ·HCl), and 41.2~45.9 g (GlcNAc).

## 3.2. Thickness of the Mucin Layer in SAMP/1 Mice and ICR Mice

Acidic glycosaminoglycans in the colonic mucus layer were dyed red-violet by PAS staining. More specifically, PAS staining dyed the mucus layer comprising goblet cells and the mucin layer that secretes mucus (Fig. 1).




×40 ×100

Fig. 1. Mucin layer dyed by PAS staining. The point indicated by arrows was measured as the mucin layer.

In SAMP/1 mice, the thickness of the mucin layer was 7.9 μm in the DW group, 9.9 μm in the GlcN ·HCl group, and 12.3 μm in the GlcNAc group (Fig. 2-A). The mucin layer of SAMP/1 mice in the

GlcNAc administration group was increased significantly as compared with the DW group (P<0.01). The thickness of the mucin layer was also higher in the GlcNAc group than in the GlcN·HCl group, but the difference was not significant.

In ICR mice, the thickness of mucin layer was 7.0 μm in the DW group, 6.2 μm in the GlcN·HCl group, and 8.4 μm in the GlcNAc group (Fig. 2-B). No significant differences were observed among the three groups, but the administration of GlcNAc increased the thickness of the mucin layer.

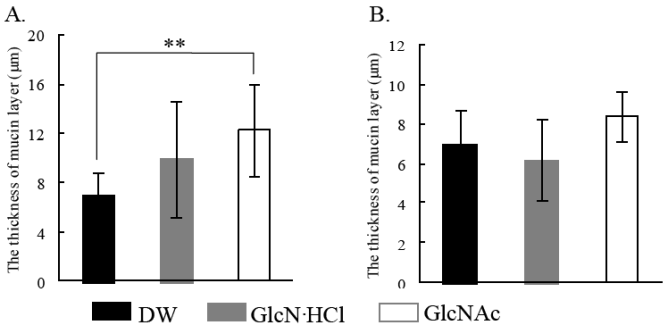



Fig. 2. The thickness of mucin layer.
A: SAMP/1 mice; B: ICR mice. **$P$<0.01.

These results were interpreted to mean that administration of GlcNAc leads to an increase in the mucin layer and the effect was greater for administration of GlcNAc than for that of GlcN·HCl. It is thought that the increase in the mucin layer due to the administration of GlcNAc enhances the guard function of the intestinal tract and improves the intestinal flora.

## 3.3. Organic Acid Concentration of Intestinal Feces in SAMP/1 and ICR Mice

In SAMP/1 mice, there were differences in the concentration of succinic acid, lactic acid, acetic acid, and n-butyric acid among the three groups (Fig. 3-A). When the concentration of acetic acid and n-butyric acid was compared between GlcNAc and GlcN·HCl administration, it was significantly lower in the GlcNAc group (P<0.05 and P<0.01, respectively). Furthermore, the concentration of n-butyric acid was significantly higher after GlcN·HCl administration than after administration of DW (P<0.05).

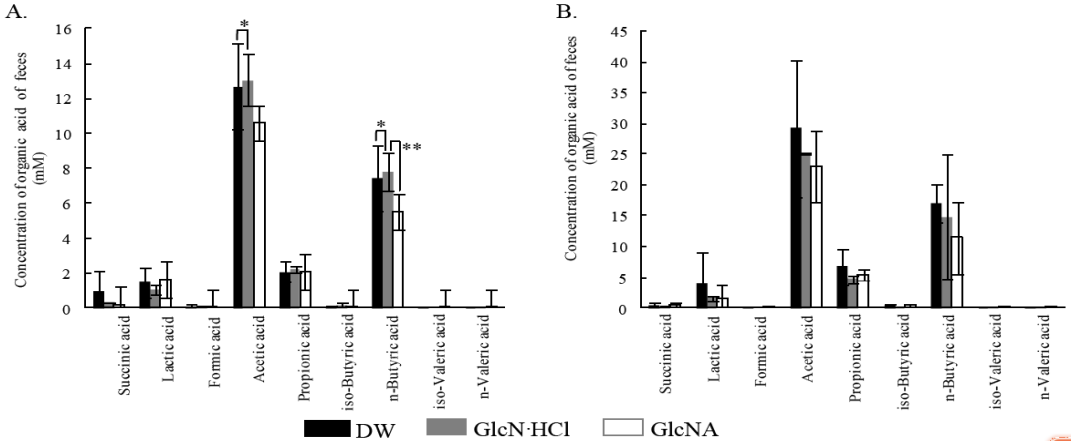



Fig. 3. Concentration of organic acid of feces
A: SAMP/1 mice; B: ICR mice. *$P$<0.05, **$P$<0.01

In ICR mice, there were differences in the concentration of succinic acid, lactic acid, acetic acid, and n-butyric acid among the three groups (Fig. 3-B). Similar to the results in SAMP/1 mice, the concentration of acetic acid and n-butyric acid showed a downward trend in the GlcNAc group in ICR mice.

It is thought that changes occurred in the organic acid concentration of the intestinal feces because the intestinal flora changed with the administration of carbohydrates. Therefore, we analyzed the intestinal flora of the mice given the different carbohydrates by T-RFLP.

## 3.4. Analysis of Intestinal Flora in SAMP/1 Mice and ICR Mice by Database Comparison

The results of T-RFLP analysis coupled with mouse intestinal database comparison for SAMP/1 mice are shown in Table 3. Clostridium and Bacteroides were observed in larger amounts after GlcNAc administration than after administration of DW. In contrast, Lactobacillus and Clostridium comprised the majority of the intestinal flora in the group with no administration of carbohydrate.

The results of the T-RFLP analysis for ICR mice are shown in Table 3. Clostridium and Bacteroides were observed in greater amounts after both GlcNAc administration and GlcN ·HCl administration than after administration of DW. In addition, Bifidobacterium was detected only in mice that had been given GlcNAc.

Both administration of GlcNAc and administration GlcN ·HCl led to diversification of the intestinal flora, and this change was greater after GlcNAc administration than after GlcN ·HCl administration. It is known that the diversity of the intestinal flora is lost during aging and that the decreasing diversity of intestinal flora is a factor of many diseases [11]. Thus, the present findings suggest that the increased diversity of the intestinal flora due to the administration of GlcNAc might improve the intestinal environment.

Two further factors were considered regarding the changes in the diversity of intestinal flora caused by the administration of GlcNAc and GlcN ·HCl. One is that the carbohydrates that were administered led to direct changes in the intestinal flora. The other is that the increase in mucin layer leads to a diversity of intestinal flora. Which is important factor to lead to changes in the intestinal flora remains to be elucidated in the future.

Table 3: Peak area ratio from T-RFLP analysis of intestinal flora

| Bacteria | SAMP/1 mice | | | | ICR mice | | |
|---|---|---|---|---|---|---|---|
| | DW | GlcN ·HCl | GlcNAc | | DW | GlcN ·HCl | GlcNAc |
| Clostridiales | 11.3 | 30.8 | 28.1 | | 14.8 | 23.9 | 13.2 |
| Clostridiales, Coriobacteriales, Erysipelotrichales | 0.6 | 0.4 | 1.4 | | 0.7 | 0.4 | 0.1 |
| Bifidobacteriales | 0.0 | 0.3 | 0.0 | | 0.0 | 0.0 | 7.0 |
| Clostridiales, Erysipelotrichales, Bacteroidales | 1.4 | 2.2 | 1.3 | | 0.8 | 1.4 | 6.8 |
| Bacteroidales | 0.1 | 0.4 | 1.0 | | 0.4 | 1.8 | 1.1 |
| Clostridiales, Bacteroidales | 1.2 | 4.4 | 6.4 | | 1.9 | 5.1 | 9.5 |
| Clostridiales, Deferribacterales, Bacteroidales | 1.9 | 3.6 | 6.0 | | 1.4 | 1.1 | 0.4 |
| Clostridiales, Lactobacillales | 82.4 | 58.9 | 53.6 | | 75.2 | 61.0 | 55.1 |
| Clostridiales, Enterobacteriaceae | 0.2 | 0.4 | 0.0 | | 0.8 | 0.4 | 3.2 |
| Others | 0.5 | 0.2 | 1.7 | | 3.9 | 5.1 | 3.5 |

## 3.5. Quantitative Analysis of *N*-acetylglucosamine Transferase C2GnT2 Expression in Elderly Mice

RT-PCR analysis was done by using a comparison quantification method. By using the β-actin gene as an internal standard, the expression level of C2GnT2 was compared among the mice given each carbohydrate and those given no carbohydrate. The expression level of C2GnT2 in the case of non-carbohydrate administration was considered to be 1.

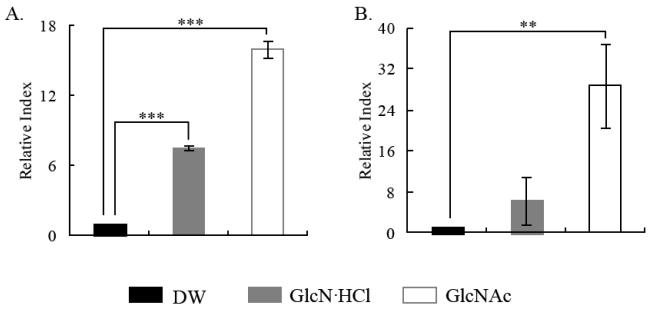



Fig. 4. Quantitative analysis of C2GnT2 by RT-PCR.
A: SAMP/1 mice; B: ICR mice. ****P*<0.001, ***P*<0.01

The quantitative analysis of C2TnG2 in SAMP/1 mice is shown in Fig. 4-A. Expression of C2TnG2 was significantly upregulated 7.5-fold after GlcN ·HCl administration and 16.1-fold after GlcNAc administration as compared with administration of DW (both P<0.001).

A similar pattern was observed in ICR mice (Fig. 4-B). The expression of C2TnG2 was upregulated 6.4-fold after GlcN ·HCl administration and 28.8-fold after GlcNAc administration, as compared with DW

administration. The difference was significant between administration of GlcNAc and administration of DW ($P<0.01$), but not between GlcN·HCl administration and DW administration.

The considerably enhanced expression of C2GnT2 observed after administration of GlcNAc or GlcN·HCl suggests that C2GnT2 is involved in the increase in the mucin layer. It has been reported that C2GnT2 knockout mice have a weakened intestinal barrier and increased susceptibility to colitis [12]. Thus, it is thought that changes in gene expression in epithelial cells due to the administration of GlcNAc or GlcN·HCl are involved in thickening of the intestinal mucin layer and enhancement of the intestinal barrier.

## 4. Conclusion

In this paper, we evaluated the effect of administration of GlcNAc and GlcN·HCl on the intestinal mucin layer and bacteria in elderly mice. The results showed that both carbohydrates increased the intestinal mucin layer, enhanced the expression of C2GnT2 in the intestinal epithelial cells, and induced diversity of the intestinal flora. The effects of GlcNAc were more pronounced than those of GlcN·HCl. As a result, it is suggested that administration of GlcNAc is beneficial for improving intestinal mucins and flora.

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