Production of α-glucosidase Inhibitor in the Intestines by *Bacillus Licheniformis*

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Research

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Abstract

Alpha-glucosidase (EC.3.2.1.20) is involved in the absorption of monosaccharides in the small intestine of animals. We aimed to find a microorganism capable of proliferating in the intestine and producing α-glucosidase inhibitor. We developed a strain capable of forming spores from dry grass and growing in an anaerobic environment was selected as *Bacillus lichenformis*. Mixing spores of this strain with a high-fat diet and high-carbohydrate diet, it was confirmed that the weight gain was significantly reduced than the high-calorie diet group without spores. Furthermore it was confirmed that *Bacillus lichenformis* administered as spores efficiently proliferated in the intestine and consistently produced α-glucosidase inhibitor by securing a constant amount of the strain and α-glucosidase inhibitor in feces after a certain period. This study shows an efficient process in which microorganisms capable of proliferating in the intestine directly produce and supply specific secondary metabolites in the intestine.

Introduction

Alpha-Glucosidase (EC.3.2.1.20) is an enzyme that decomposes oligosaccharides and disaccharides into monosaccharides in the small intestine, as key enzyme that plays an important role in the digestion and absorption of saccharides (Leroux-Stewart et al. 2004; Schmidit et al. 1977). In the intestine, α-Glucosidase inhibitor (AGI) is effective in preventing postprandial hyperglycemia by inhibiting α-glucosidase activity and delaying the absorption of sugar, so it is used as a representative treatment for type II diabetes (Leroux-Stewart et al. 2004; Van de Laar et al. 2005; Chen et al. 2005; Zhang et al. 2013). For this purpose, many efforts have been made to develop an effective and safe natural substance-derived AGI to relieve diabetes and control the absorption of monosaccharides (Gunawan-Puteri & Kawabata 2010; Ji et al. 2010). Among natural products, The AGI of plant origin are known; include aza sugars such as 1-deoxynojirimycin in mulberry leaves (Tao et al. 2013; Akira et al. 2011), isoflavones (Choi et al. 2008) in soybeans such as genistein (Lee & Lee 2001), daidzein (Park et al. 2013), some flavone glycosides (Tadera et al. 2006), catechin (Gamberucci et al. 2006), polyphenols (Kumar et al. 2011) such as kaempferol (Peng et al. 2016), salacinol (Xie et al. 2011), anthocyanin (Ifie et al. 2018) of hibiscus origin, etc. The AGI of microbial origin are known; pseudo-oligosaccharide (Truscheit et al. 1981; Lee et al. 1997) from *Actinoplanes* strain, 1-deoxynojirimycin (Onose et al. 2013; Paek et al. 1997; Hardick et al. 1992) from *Streptomyces, Bacillus* strain, etc.

The AGI originating from aerobic microorganisms such as *Streptomyces* and *Bacillus* can be obtained through culture. It has the advantage of producing AGI in large quantities in a short period through the breeding of high-producing strains and optimization of the culture process (Zhang et al. 2019; Lee et al. 2018). However, the AGI of microbial origin is difficult to use into originals cause various factors such as fermentation odor and concentration of effective substances, there is a need to undergo purification (Zhu et al. 2013; Zhu et al. 2008).

The intestinal microbiota such as lactic acid microorganism proliferate in the intestine and exhibit various physiological effects that control the inflammatory response of the host or produce useful

substances such as secondary metabolites and also generate energy for use by the host through metabolic processes (Wang et al. 2019; Riedl et al. 2017; Nieuwdorp et al. 2014; Sweeney & Morton 2013). This means that if microorganisms that are capable of growing in the anaerobic part of intestine produce large amounts of secondary metabolites, the possibility of using the intestine as a production site and using space for specific secondary metabolites is high.

Several specific microorganisms, such as *Bacillus* and *Streptomyces* are known to produce large amounts of AGI in an aerobic environment (Onose et al. 2013; Paek et al. 1997; Hardick et al. 1992), however they are difficult to proliferate in the intestine which is an anaerobic environment, they are difficult to supply AGI in the intestine.

In this study, a new strain that is capable to proliferate in the intestine for intestinal production of useful substances and capable of producing large amounts of AGI in an anaerobic environment such as the intestine was explored, a new strain was examined that is capable of intestinal proliferation, production of AGI in the intestine and weight loss effect accordingly.

Results And Discussion

Screening strain

About 400 pieces of rice straw and hay collected from all over the world such as Korea, China, Japan and the United States were used as samples to isolate microorganisms. A small amount of sterile saline is added to each sample and suspended, and the spore solution obtained by heat treatment in an 80℃ water bath for 20 minutes is used in an LB plate medium containing 2% agar (1% tryptone, 0.2% sucrose, 0.5% yeast extract and 0.5% NaCl, pH 7.0) and anaerobically cultured in an incubator at 55℃. for two days to isolate colony-forming microorganisms. Single colonies obtained by separating from hay were inoculated in 5 mL of 5% soy flour suspension medium and cultured with shaking at 37℃ for 24 hours, and then 60 species with high AGI activity in the supernatant were selected. The selected 60 strains were capable of growing for anaerobic growth at 50℃ and three strains with high AGI activity were selected as strains that were capable of using propionic acid. The selected three strains were identified as *Bacillus licheniformis* as a result of classification by taxonomic characteristics (Table 2) and analysis method according to Manual of Systematic Bacteriology (Bergey 2001).

Table 2. *Bacillus licheniformis* NY1505 physiological specificity.

Gram staining	+	Acid from	
		D-Glucose	+
		L-Arabinose	+
55℃ growth	+	D-Xylose	+
Anarobic growth	+	D-mannitol	+
Propionate utilization (3%)	+	Hydrolysis of	
Citrate utilization	+	Casein	+
Nitrate redued to nitrite	+	Gelatin	+
Voges-Proskauer test	+	Starch	+

The microorganism with the highest AGI production capacity was finally selected and to increase the AGI production capacity of the strain after inducing mutation by treatment with NTG (100μg/mL) to reach 99.9% kill rate, spread to 200-300 per sheet on L-broth plate medium and incubate anaerobically for two days at 37℃. The resulting colonies were randomly inoculated in 5 percent soybean flour medium and cultured with shaking at 40℃ for two days, and then the centrifuged supernatant was measured for the AGI activity. By repeating the mutation twice in the same way, a strain having high AGI activity was selected and named *B. licheniformis* NY1505. As a result of analyzing the 16s RNA nucleotide sequence of *B. licheniformis* NY1505 strain, it showed high homology with *B. licheniformis.* The dendrogram shows that *B. licheniformis* NY1505 (the accession number KCTC13021BP) is allied species with the *B. licheniformis* type strain (Fig. 1).

Analysis of AGI

5 x 10^5 spores of NY 1505 were inoculated into 500g of steaming soybeans and covered the film, incubated at 37℃ for 24 hours, added 2.5L of 70% (v/v) ethanol, extract twice, and evaporated under reduced pressure. To 150 mL of the concentrated extract, 300 mL of hexane, dichloromethane, and Ethyl acetate were sequentially added twice, stirred for 2 hours, allowed to stand for 2 hours, and fractionated to wash the aqueous layer. After drying the aqueous layer, 100 mL of 90% ethanol was added to dissolve it, followed by silica gel column (100 mL) chromatography. The mobile phase was stepwise gradient from 1:1 solution of acetonitrile and methanol to 3 : 2 solution (Total 1000 mL). The AGI activity of each fraction was measured to obtain two fractions, AGI 1 and AGI 2. AGI 1 and AGI 2 each appeared as a single spot in thin layer chromatography, and the structure was determined through NMR analysis.

The chemical shift of AGI 1 is as follows.

^{1}H-NMR (400 MHz C_5D_5N) δ_H, *J* (Hz)$^{\alpha}$ = 4.71 (d, 1H, *J* = 1.8 Hz, H29a); 4.54 (d, 1H, *J* = 1.8 Hz, H-29b); 3.88 (d, 1H, *J* = 11.2, 4.4 Hz, H-11); 1.60 (s, 3H, H-30); 1.06 (s, 3H, H-25); 1.03 (s, 3H, H-23); 1.00 (s, 3H, H-24); 0.98 (s, 3H, H-27); 0.97 (s, 3H, H-26).

^{13}C-NMR (100 MHz, C_5D_5N) δ_C = 216.1 (C-3); 169.4 (C-28); 148.1 (C-20); 108.1 (C-29); 68.2 (C-11); 59.7 (C-5); 59.4 (C-17); 52.7 (C-9); 42.7 (C-18); 41.8 (C-14); 41.7 (C-19); 40.8 (C-8); 39.5 (C-4); 38.5 (C-1); 34.1 (C-10,13,22); 37.1 (C-7); 33.8 (C-16); 30.1 (C-15); 28.5 (C-21); 26.9 (C-12); 26.7 (C-2); 24.7 (C-23); 19.4 (C-6); 17.8 (C-25); 19.5(C-26); 15.4 (C-24,30); 12.4 (C-27).

AGI 1 is a triterpene-type substance in which five rings are connected and has a structure similar to betulinic acid. The chemical structure was found to be 3-oxo-11α-hydroxy-lup-20(29)-en-28-oic acid.

The inhibition pattern of AGI 1 through the Lineweaver-Burk plot was confirmed as non-competitive inhibition (Fig. 2).

AGI 2 was presumed to be 1-deoxynojirimycin (DNJ), and as a result of running NMR, DNJ and NMR results were confirmed to be consistent. The chemical shift of AGI 2 is as follows.

^{1}H-NMR (400 MHz D_2O) δ_H, *J* (Hz)$^{\alpha}$ = 3.96(d, 1H, *J* = 12.7, 3.5 Hz, H-6a); 3.89 (d, 1H, *J* = 12.8, 5.5 Hz, H-6b); 3.78 (m, 1H, H-2); 3.65 (1H, t, *J* =10 Hz, H-4); 3.52 (t, 1H, *J* = 9.5 Hz H-3,); 3.52 (s, 1H, *J* = 12.7, H-1a); 3.19 (m, H, H-5) 3.15 (s, 1H, *J* = 12.8, H-1b).

^{13}C-NMR (100 MHz, D_2O) δ_C = 78.1 (C-3); 70.2 (C-4); 69.4 (C-2); 62.2 (C-5); 59.8 (C-6); 48.5 (C-1).

AGI 2 showed a tendency to inhibit competitive inhibition (Fig. 3).

Animal experiment

High carbohydrate diet

After starting the diet and measuring the weight of each group every week, the lowest and highest values were excluded and statistically processed. In addition, more than 3g of fresh feces were collected for each cage to examine the number of microorganism.

The high carbohydrate diet group showed a weight gain rate about 130% compared to the standard diet group in week 4, but the high carbohydrate diet group with spores of the *B. licheniformis* NY1505 strain showed the weight gain rate of about 90% compared to the standard diet group(Fig 4).

High fat diet

The high fat diet group showed a weight gain rate about 150% compared to the standard diet group in week 6, but when the high fat diet group with spores of the *B. licheniformis* NY1505 strain showed the weight gain rate about 120% compared to the standard diet group(Fig. 5).

Comparing the results in Fig. 4 and 5 the high fat diet group was more sensitive than the high carbohydrate group to the administration of *B. licheniformis* NY1505 spores at the week 3 or 4. In particular, it has been reported that when DNJ, a component of AGI 2, is administered for a long period of 12 weeks or longer, it activates β-oxidation, which decomposes fatty acids in mitochondria, and inhibits liver fat formation (Tsuduki et al. 2013; Tsuduki et al. 2009). Therefore, this is expected to be because *B. licheniformis* NY1505 proliferates in the intestine and produces AGI that activates β-oxidation, which is the catabolic action of fatty acids. It is known that betulinic acid, which has a similar structure to AGI 1, also inhibits adipogenesis by inhibiting differentiation in adipocyte growth (Mohsen et al. 2019).

The number of *B. licheniformis* NY1505 microorganism excreted in feces

When fecal extract samples with sterile saline are anaerobically cultured at 55℃ in a medium containing 3 percent propionate, only *B. licheniformis* grows selectively. So the surviving strain in this culture condition were determined as *B. licheniformis* NY1505. A mouse ingested about 5 x 10^5 spores per day from the feed. Three weeks after spore administration, about 10^7 cells of *B. licheniformis* NY1505 were detected per 1g of feces. This strain proliferates vigorously in the intestine and is excreted (Fig. 6). Spores were administered for seven weeks and the number of microorganism rapidly decreased at 8th week when not administered for one week. It can be judged that it inhabits temporarily without adhering to the intestine.

The number of α-glucosidase inhibitor NY1505 microorganism excreted in feces

The B. licheniformis NY1505 strain proliferates vigorously in the intestine and produces AGI. From the 2nd week when the *B. licheniformis* NY1505 strain which had proliferated in the intestine was being detected in the feces, AGI that produced in the intestine was excreted into the feces equally (Fig. 7). As shown in Fig. 6, the amount of *B. licheniformis* NY1505 excreted is stabilized, and the amount of AGI excreted is also constant from 4thweek to 7th week. In other words, AGI is always continuously produced in the intestine, its concentration is maintained and a constant amount of the produced AGI is excreted. Considering that the AGI activity of natto produced from the *B. licheniformis* NY1505 strain is 90-95 units/g (data not shown), it can be seen that a significant amount of AGI is produced in the intestine and some of it is excreted.

Conclusions

Gut microbiota inhabiting in the intestine proliferate in the intestine and produce secondary metabolites (Kopp-Hoolihan 2001). Secondary metabolites produced by the gut microbiota may affect the host depending on the amount. In other words, various physiological activities are capable to expect by intentionally administering gut microbiota that can produce a large amount of useful secondary metabolites (Parvez et al. 2006; Kopp-Hoolihan 2001). In this study, we investigated the possibility of intestinal production of physiologically active substances as AGI that involved in the absorption of sugars in the digestive tract by microbiota capable of proliferating in the intestine.

The conditions for the strains to produce physiologically active substances in the intestine should be safe, they should be able to proliferate in the anaerobic environment in the intestines, they should form spores to reach the intestines efficiently, the high productivity of physiologically active substances in the anaerobic environment (Parvez et al. 2006; Kopp-Hoolihan 2001). Therefore, strains were screened from the nature. As a result *B. licheniformis* NY1505 was obtained. *B. licheniformis* NY1505 efficiently reaches the intestine in the form of spores, then proliferates, produces a large amount of AGI, and exhibits the physiological activity of the host that slows the rate of weight gain (Figs. 4, 5).

On the other hand, how the administered microbiota adheres and inhabits in the intestine depends on the need for supply of the secondary metabolite. In other words, it is preferable to inhabit and produce the secondary metabolite in the intestine only for a necessary period. As shown in Fig. 6, following the intestinal habitat of *B. licheniformis* NY1505, the numbers of strain excreted out of the body decreases after the administration of the strain is finished. *B. licheniformis* NY1505 inhabits and is excreted in a relatively short time rather than adhering and inhabiting in the intestine, so it is capable to use only for a necessary period.

It takes a lot of time and cost to process such as extract, purify, and drying physiologically active substances from fermented products of animals and plants or microorganisms (Zhu et al. 2013; Gunawan-Puteri & Kawabata, 2010; Ji et al. 2010; Zhu et al. 2008). In the case of extracts, there are many factors that lower processing efficiency, such as low concentration of active substances, color derived from raw materials and odor. To overcome this problem, it takes time and cost must be invested (Sun et al. 2019; Vásquez et al. 2014).

In this study, it is shown an efficient process of producing and supplying secondary metabolites directly in the intestine by administering strain capable of proliferating in the intestine. AGI is a compound that works inside the intestine, but it is expected that this process will work. This study suggests that by oral administration of microbiota that is capable of intestinal proliferation, the intestinal environment is used as a factory to produce secondary metabolites. It can be a new supply and intake method for physiologically active substances.

Methods/experimental

Materials

Reagents such as N-methyl-N'-nitro-N-nitrosoguanidine (NTG), ρ-nitrophenyl α-D-glucopyranoside(pNPG), sodium propionate, potassium phosphate, and sodium carbonate were used with guaranteed reagents.

Alpha-glucosidase Inhibition Assay

Mix 30 μL of the sample, 25 μL of α-glucosidase enzyme solution (1 unit), and 25 μL of 0.1M potassium phosphate buffer (pH 7.0) to each well of the microplate, pre-incubate for 10 minutes at 37°C, and then

add 50 μL of 3mM ρNPG at 37℃, incubate for 20 minutes.

The reaction was stopped by adding 100 μL of 0.1M sodium carbonate, and the inhibition rate was measured by substituting the absorbance at 405 nm into the following equation (Kim et al. 2005).

The inhibition rate = (A-B)/A × 100

In the above equation, A is the absorbance of the control and B is the absorbance of the sample containing the inhibitor. Inhibition rate 1 unit was defined as the amount of inhibitor when 100% inhibition of α-glucosidase used in the assay.

Animals

Experimental animals were bred in research facilities. Three-week-old ICR mice were used as experimental animals after the acclimation period before the experiment were classified by weight equally and classified into ten animals per group and 5 per cage. The illuminance was controlled by turning it on every 12 hours and the temperature of the breeding room was adjusted to 21℃. The feed composition of each group is as shown in Table 1, and the high carbohydrate group and the fat group was reared by dividing the spores administered group and the non-administered group, respectively.

Table 1
Composition of animal experimental feed.

	Standard (g)	High carbohydrate (g)	High fat (g)
Carbohydrate	30	75	39
Protein	50	12	12
Fat	5	10	46
Total kcal	365	438	618

The spore administration diet was prepared by directly mixing 10^8 cells of spores per 1 kg of feed. During the breeding period, body weight was measured, feces were collected and the amount of microorganism contained in the feces and excreted and the activity of AGI were measured at regular intervals. This study was approved by the Animal Care and Use Committee of Kangwon National University (permit no. KW-190103-11)

Statistical analysis

The results of each experiment were expressed as the mean with standard deviations (± SD). A one-way analysis of variance (ANOVA) test (Bonferroni, SPSS, v.32, for Windows) was performed to determine the group means. Values were considered to be significant when P was less than 0.05($P \leq 0.05$).

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

All data generated or analysed during this study are included in this published article and its supplementary information files.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

HI Rhee conceived of the presented idea. HW Kim developed the theory and performed the computations. HW Kim, DC Lee verified the analytical methods. HW Kim wrote the manuscript with support from DC Lee. HI Rhee encouraged HW Kim to investigate a specific aspect and supervised the findings of this work. All authors discussed the results and contributed to the final manuscript.

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Figures

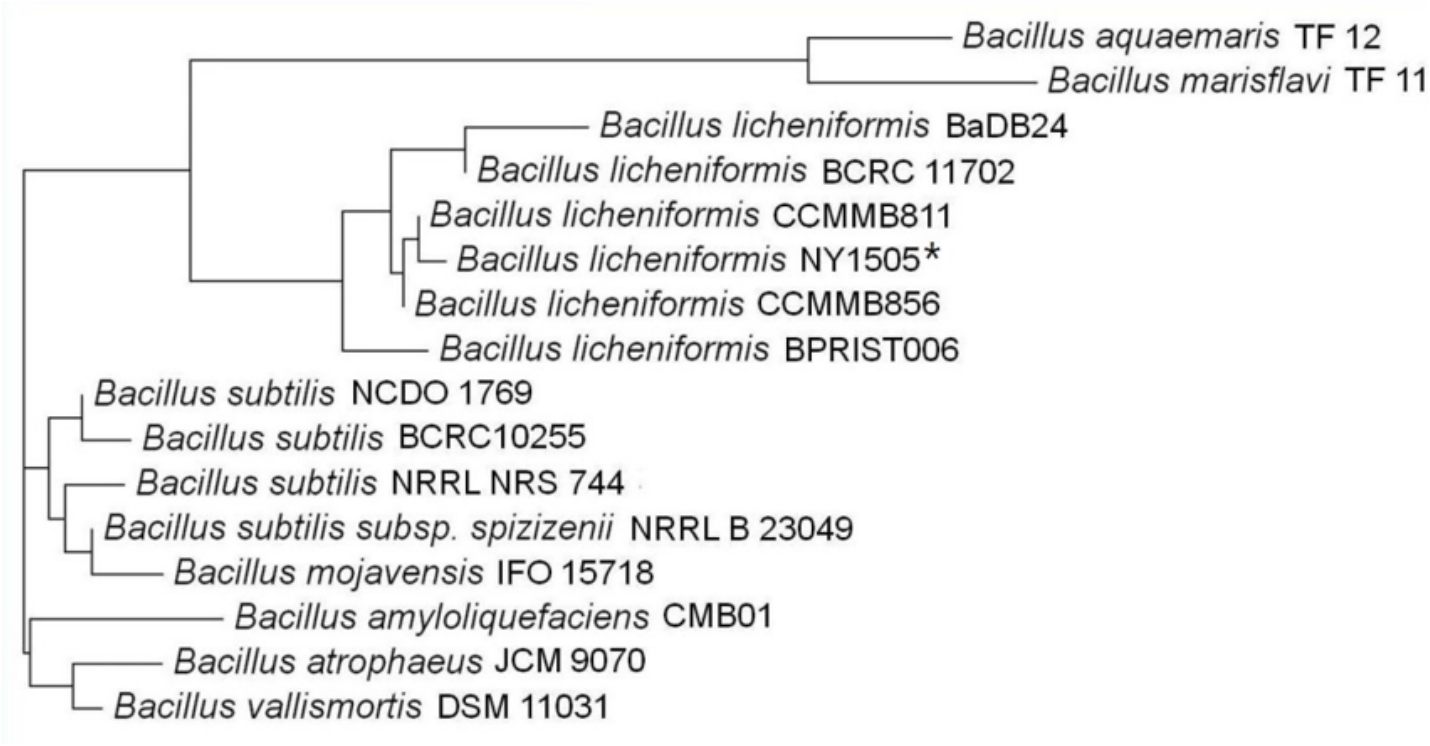
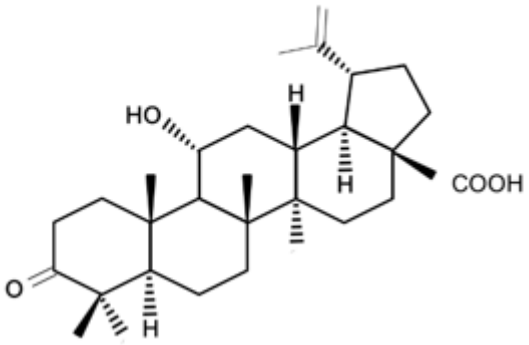


Figure 1

Phylogenetic tree highlighting the taxonomic relation of B. licheniformis NY1505 strain based on 16S rDNA amplicon within the Bacillus clad (Sequence accession number LY517136)

a



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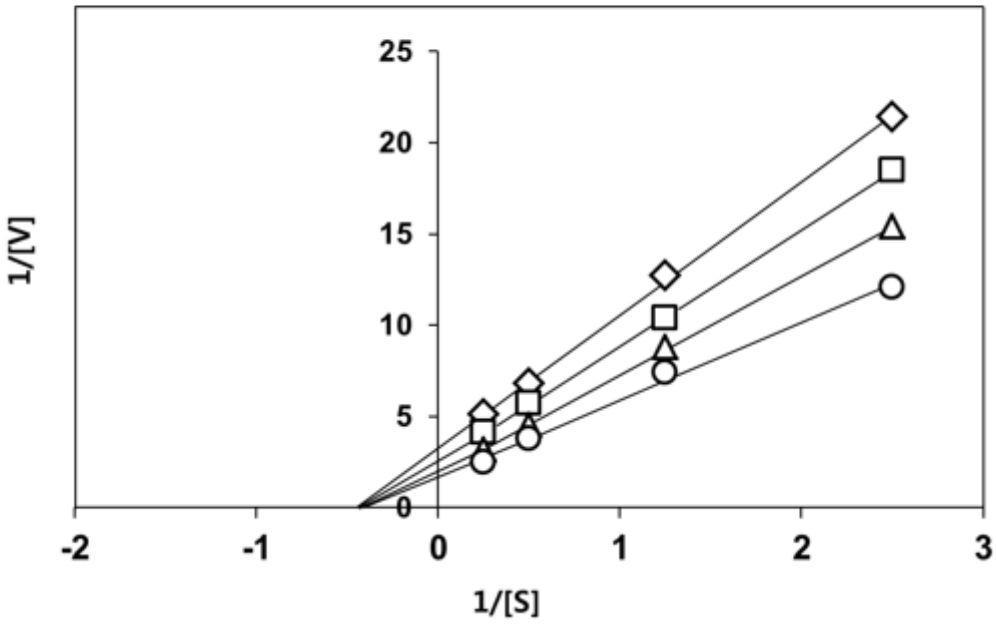

Figure 2

Chemical structure of AGI 1 and the enzyme inhibition type of AGI 1 a Chemical Structure of AGI 1 b Inhibition type of AGI 1 showed non-competitive inhibition by Lineweaver - Burk plot. Each symbol showed amounts of AGI 1. ▯ is 1140 μg, □ is 570 μg, △ is 228 μg, ● is no inhibitor

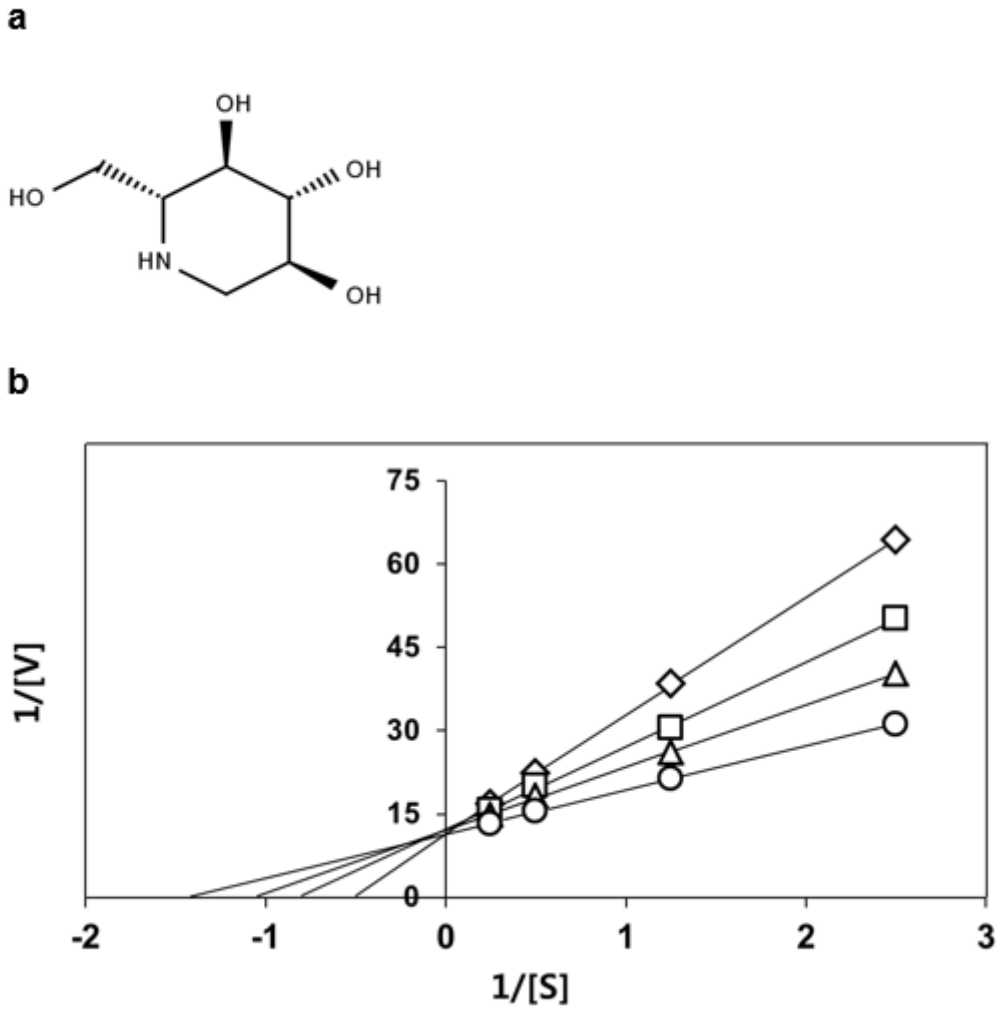

Figure 3

Chemical structure of AGI 2 and the enzyme inhibition type of AGI 2 a Chemical Structure of AGI 2 b Inhibition type of AGI 2 showed competitive inhibition by Lineweaver - Burk plot. Each symbol showed amounts of AGI 2. ◇ is 48.9μg, □ is 32.6μg, △ is 16.3μg, ● is no inhibitor

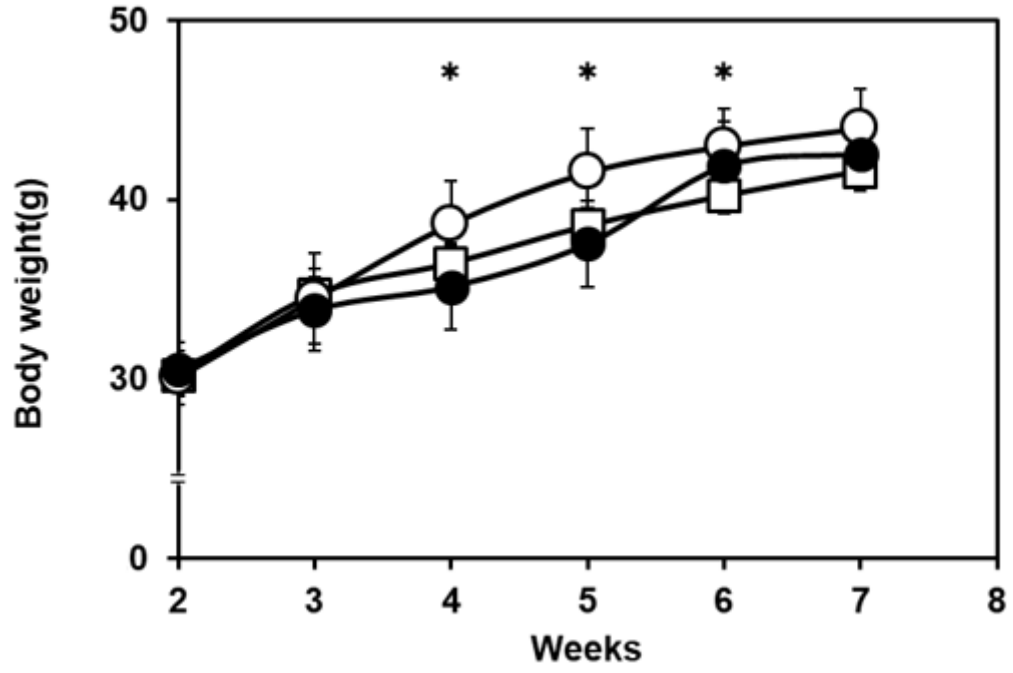

Figure 4

Comparison of weight alteration in High carbohydrate diet clade with a standard diet Each symbol □ showed weight alteration of standard diet group, ● showed weight alteration of high carbohydrate diet group, ● showed weight alteration of high carbohydrate diet with NY1505 spore group. Values were considered to be significant (□) when P was less than 0.05($P \leq 0.05$). □ meant that high carbohydrate diet group with and without NY1505 showed to be accompanied reliability meaning significant.

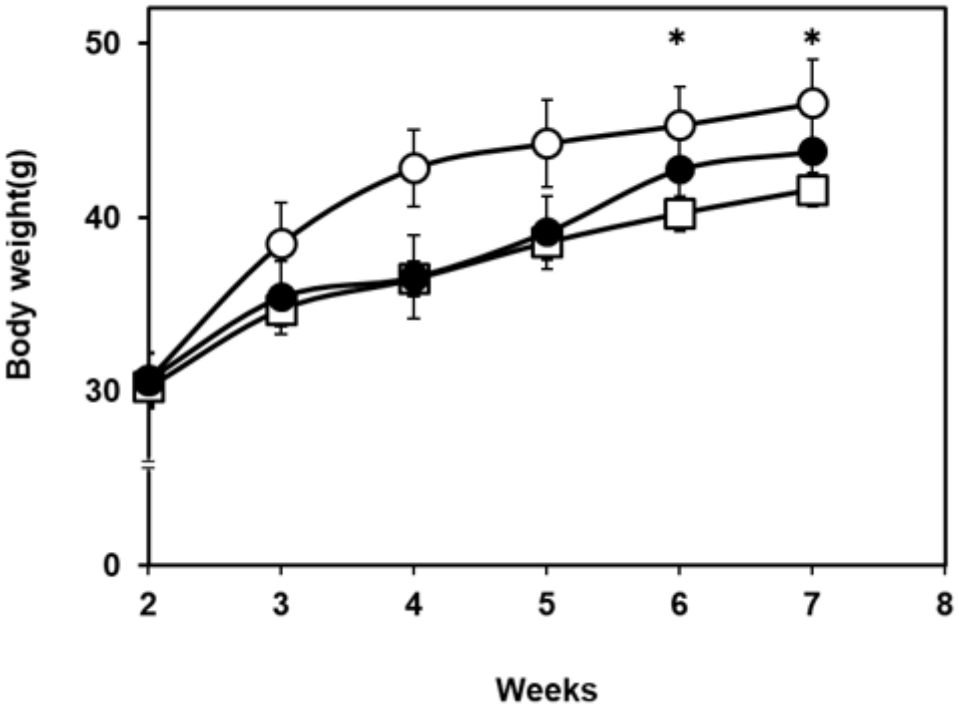

Figure 5

Comparison of weight alteration in fat diet clade with a standard diet Each symbol □ showed weight alteration of standard diet group, ● showed weight alteration of fat diet group, ● showed weight alteration of fat diet with NY1505 spore group Values were considered to be significant (□) when P was less than 0.05($P \leq 0.05$). □ meant that high fat diet group with and without NY1505 showed to be accompanied reliability meaning significant.

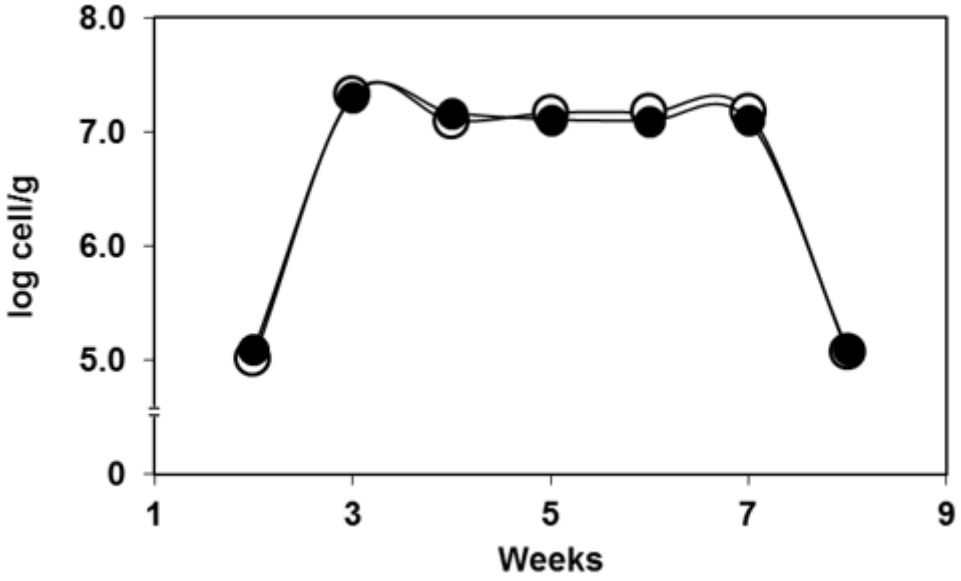

Figure 6

A secreting amount of NY 1505 CFU per 1g from feces Each symbol showed secreting amount of NY 1505 CFU per 1g from feces. ● meant high carbohydrate diet with NY1505 spore group, ● meant high fat diet with NY1505 spore group.

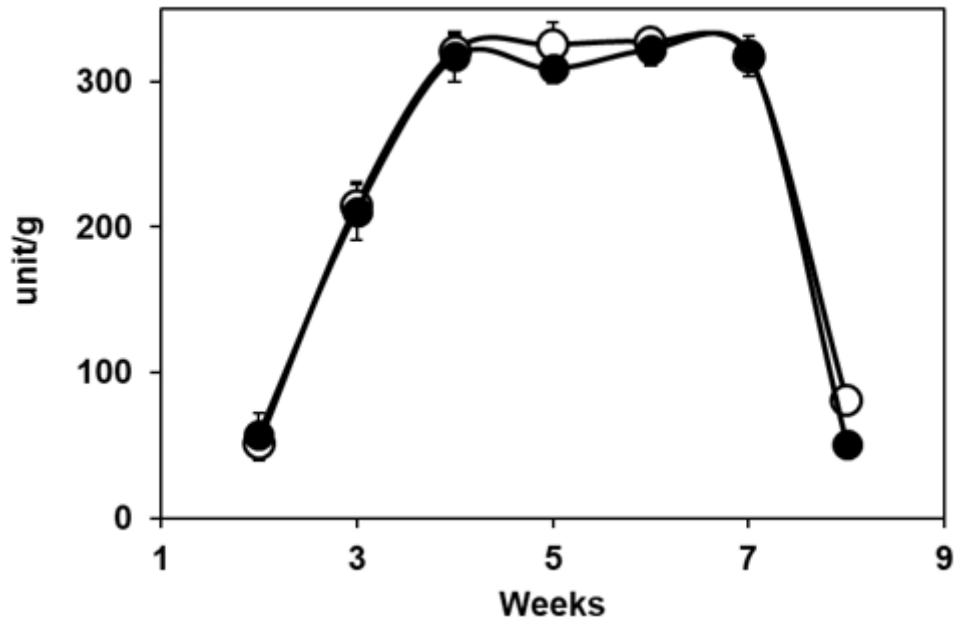

Figure 7

A secreting amount of AGI per 1g from feces Each symbol showed a secreting amount of AGI per 1g from feces. ● meant high carbohydrate diet with NY1505 spore group, ● meant fat diet with NY1505 spore group

Supplementary Files

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- GABPBR.jpg